Ecological and physiological studies on freshwater autotrophic picoplankton

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by

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This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

G.R.W. Hawley

Abstract

A series of studies were conducted to ascertain the importance of autotrophic picoplankton (0.2 - 2.0 μm in the longest dimension) in freshwaters. These included surveys of populations in lakes and rivers, and experiments on strains which had been isolated in clonal, axenic culture.

Methods were developed to preserve, count and identify autotrophic picoplankton. Epifluorescence microscopy was used routinely throughout the study and it was shown that cells could be preserved in buffered formalin or glutaraldehyde for at least 12 months without a loss in cell count. Autotrophic picoplankton fluoresced red or orange depending on the photosynthetic pigments present, and a method based on fluorescence characteristics was developed to distinguish cyanobacterial from eukaryotic cells. Eukaryotes fluoresced most intensely under blue excitation light and were barely visible under green excitation light and cyanobacterial cells had the reverse response. In addition, fluorescence from eukaryotic cells faded faster than the fluorescence from cyanobacterial cells.

Samples from standing waters from around the world were collected from 5 continents in a survey to discover how widespread autotrophic picoplankton are in the world; densities ranged from 1.02×10^2 from a pool in Saudi Arabia to 1.20×10^6 in Ennerdale Water in the UK. A survey of 30 lakes in northern England and Scotland revealed autotrophic picoplankton in every sample. Cell densities ranged from a minimum of 1.02×10^2 in Esthwaite Water to a maximum of 4.26×10^5 in Ennerdale Water. In two mountain streams the only autotrophic picoplankton found were aberrant.

Studies on the seasonality of autotrophic picoplankton from 10 UK lakes showed that population densities differed by at least two orders of magnitude in a year. In every lake densities reached a maximum at mid- to late summer and highest densities were found in nutrient-poor waters. There was a marked difference in the relative abundance of orange and red fluorescing cells in all lakes.

A number of standard methods were used to isolate and purify strains of autotrophic picoplankton, including a novel method using a laser flow cytometer to purify bacterized unialgal cultures. 14 strains were studied (9 obtained by the author) and they demonstrated different growth rates when grown with different nitrogen substrates and when grown under differrent photon flux density. Twelve strains showed cell-bound phosphomonoesterase activity at pH 10.3 and nine strains showed cell bound phosphodiesterase activity at pH 10.3. No strain showed evidence of nitrogen fixation, chemoheterotrophic growth, desiccation tolerance, motility nor chromatic adaptation.

Abbreviations

°C	degrees Celsius
g	gramme
mg	milligramme
ml	millilitre
µl	microlitre
m	metre
µm	micrometre
d	day
min	minute
s	second
h	hour
M	molar
mM	millimolar
d.wt	dry weight
P	phosphorus
TFP	total filtrable phosphorus
APA	alkaline phosphatase activity
pNPP	p-nitrophenyl phosphate
bis-pNPP	bis(p-nitrophenyl)phosphate
HEPES	N-2-hydroxymethylpiperazine-N-2'-sulphonic acid
PAR	photosynthetically active radiation
n	number of samples
p	probability
PC	phycocyanin
PE	phycoerythrin
chl a	chlorophyll a
CV	coefficient of variation
DWR	deepwater rice
μ_{max}	maximum specific growth rate
t_d	doubling time
ATCC	American Type Culture Collection
CCAP	Culture Collection of Algae and Protozoa
CCMP	Center for Culture of Marine Phytoplankton
SAG	Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universitat Göttingen
SMBA	Scottish Marine Biological Association
UTEX	University of Texas Culture Collection of Algae

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CHAPTER 1

INTRODUCTION

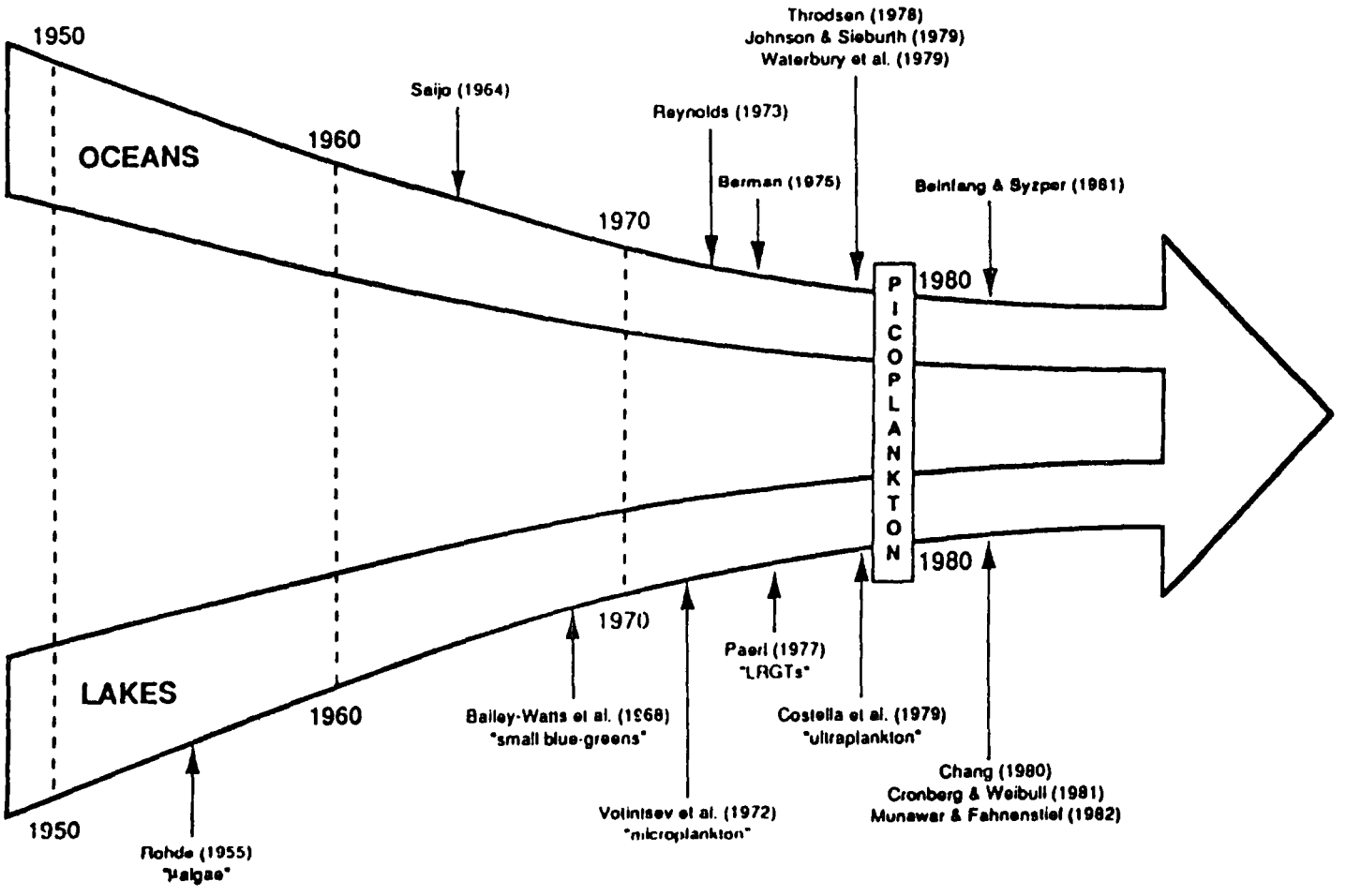
1.1 INTRODUCTORY REMARK

Autotrophic picoplankton became the focus of detailed studies in oceans as a result of two widely quoted papers in the late 1970s (Waterbury et al., 1979; Johnson & Sieburth, 1979). Marine studies have tended to be predominant, at least until recently, and the following therefore reviews some of the marine literature.

1.2 GENERAL INTRODUCTION

The term picoplankton was coined in 1978 by Sieburth et al. as organisms in the size range 0.2 - 2.0 μm. One year later independant observations by Johnson and Sieburth (1979) and Waterbury et al. (1979) noted the abundance of marine photoautotrophic organisms that fell within the picoplankton size range. Since these first observations of photosynthetic picoplankton in the ocean, there have been numerous reports of autotrophic picoplankton from ocean provinces around the world. A few years after their formal recognition in marine environments, reports of freshwater autotrophic picoplankton started to appear in the literature (eg Chang, 1980; Cronberg & Weibull, 1981).

Fig. 1.1 Historical observations of marine and freshwater autotrophic picoplankton (Stockner, 1988)



1.3 HISTORICAL OBSERVATIONS

Although formally recognised for the first time in 1979, isolated observations of small photosynthetic organisms in the plankton (marine and freshwater) have been documented for many years. Lohmann (1911) noted small green cells which passed through plankton net mesh (35 μm) but were present in centrifuged pellets. Rodhe (1955) described similar cells from Swedish lake samples as "μ-algae". One of the few recorded blooms of these bacterial-sized photosynthetic organisms was noted by Bailey-Watts and Bindloss (1968) in Loch Leven. Votinsev et al. (1972) recorded small photosynthetic organisms in Lake Baikal and named them "ultraplankton". Paerl et al. (1977) reported similar organisms in the southern hemisphere and called them LRGTs (Little Round Green Things) reflecting their unknown taxonomic status. Historical observations are summarized in Fig. 1.1. Despite isolated reports, the reasons for the oversight of this abundant phototrophic mass from routine plankton surveys of marine and freshwater environments were twofold: firstly, early surveys were carried out by collecting cells retained by fine-meshed nets, the minimum aperture diameter of which was around 35 μm; picoplankton-sized cells would pass straight through nets. Secondly, autototrophic picoplankton-sized cells are indistinguishable from heterotrophic bacteria under the light microscope; it is likely that many researchers scored autotrophic picoplankton as heterotrophic bacteria (eg J.W.G. Lund, personal communication). The major technological advance leading to formal discovery of the group was the

availability of epifluorescence microscopy. Hobbie et al. (1977) described a method for detecting aquatic bacteria on Nuclepore polycarbonate membranes by filtration, after staining aliquots of sample water with a fluorochrome and using epifluorescence to count fluorescing cells. Both Johnson and Sieburth (1979) and Waterbury et al. (1979) used adaptations of this method, exploiting autofluorescent properties of photosynthetic pigments, to detect phototrophic picoplankton. More recently, laser flow cytometry has proved a useful tool for quantifying picoplankton populations (Li, 1987).

1.4 DEFINITION AND NOMENCLATURE

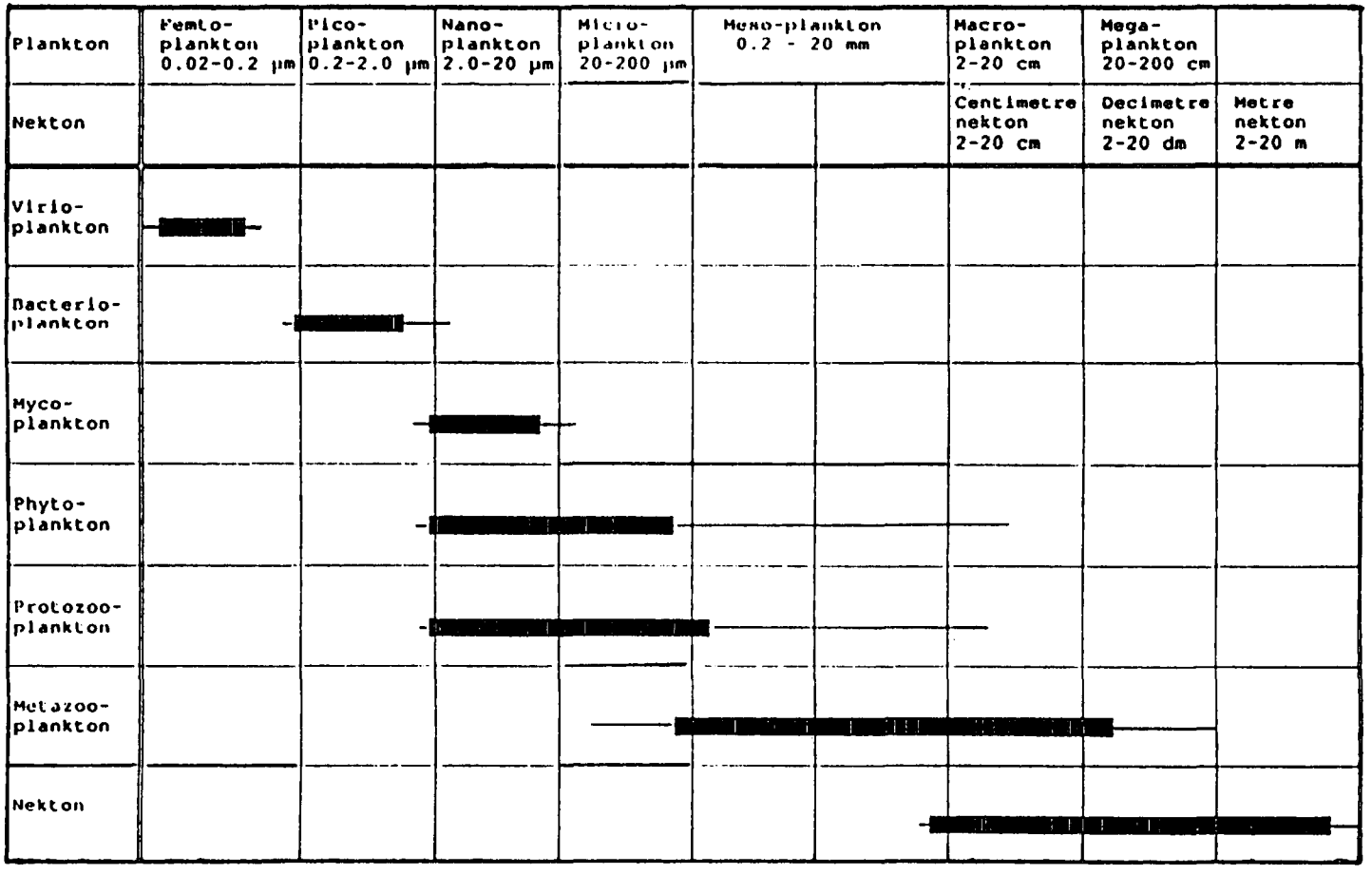
Sieburth et al. (1978) defined picoplankton as organisms in the size range 0.2 - 2.0 μm; it was based on a logarithmic scale describing pelagic size classes from viral particles to large nekton (Fig. 1.2). Picoplankton thus includes both phototrophic and heterotrophic organisms; however since some bacteriologists preferred to retain the term bacterioplankton to describe the latter (eg Fuhrman et al., 1980; Ammerman et al., 1984; Hagström et al., 1984) it became customary to use the term picoplankton only for phototrophic micro-organisms in this size range. Sicko-Goad and Stoermer (1984) proposed a new scale to cover pelagic size classes based on decimal dimensions (Table 1.1).

Table 1.1 Proposed terminology and size ranges for phytoplankton cell sizes (after Sicko-Goad & Stoermer, 1984).

Femtoplankton	0.01	-	0.1	μm
Picoplankton	0.1	-	1.0	μm
Nanoplankton	1.0	-	10.0	μm
Microplankton	10.0	-	100	μm

The lack of willingness by scientists to adopt a standard nomenclature has

Fig. 1.2 Size classes of pelagic organisms based on Sieburth's classification (Sieburth, 1978)



resulted in a variety of definitions to describe picoplankton in order to distinguish the autotrophic from the heterotrophic component. Some authors adopt picoplankton as a particle size and qualify this with epithets such as autotrophic or photoautotrophic or by inserting "phyto" to coin the term picophytoplankton (Li et al., 1983; Takahashi & Hori, 1984; Smith et al., 1985). In nature there is a continuous spectrum of particle size and Murphy and Haugen (1985) point out that very small plankters in the dimensional interval 0.5 - 8.0 μm are numerically dominant in marine environments. A combination of this observed spectrum and the difficulty of accurately separating size fractions by filtration has led to a wide interpretation of picoplankton size class in the literature. This in turn makes comparison of a growing body of literature very difficult (Table 1.2).

Table 1.2 Picoplankton size classes in the literature.

Author	Size
Bienfang et al. (1984)	<3 μm
Craig (1986)	<2 μm
Fogg (1986)	"Pass through filter with 2 μm pores but not one with 0.2 μm pores"
Glover et al. (1985)	<3 μm >0.2 μm
Glover et al. (1986)	<2 μm
Iturriaga & Marra (1988)	<3 μm
Lewin & Cheng (1985)	1-5 μm

1.5 CLASSIFICATION

1.51 Prokaryotic autotrophic picoplankton

All identified autotrophic picoplanktonic cyanobacteria have been assigned to the order Chroococcales reserved by taxonomists for unicellular species dividing by binary fission in one or more planes at right angles to one another (Stanier et al., 1971). The taxonomic classification of

cyanobacterial picoplankton has been difficult for two reasons: a) the small size of organisms reveal few taxonomic markers under light or epifluorescence microscopy; b) the more general debate whether "blue-greens" should follow the botanical code or bacteriological code of nomenclature.

Waterbury and Rippka (1989) provide the most recent classification of the Chroococcales in Bergey's Manual of Systematic Bacteriology (1989). The definition used here corresponds to "Section I" of Rippka et al. (1979). Within the Chroococcales the Synechococcus group is a provisional assemblage which is loosely defined as:

"unicellular coccoid to rod-shaped cyanobacteria that divide by binary fission in a single plane. The cells are 3 μm in diameter, contain photosynthetic thylakoids located peripherally, and lack structured sheaths".

There are six clusters within the group with representative strains (Table 1.3). Clusters are equivalent to genera but have not been formally recognized because they are based on an incomplete data set with a relatively limited number of phenotypic properties; thus formal delineation of genera and species will have to await further phenotypic and genetic analyses. In the absence of species level identification, it is common practice to use clone or isolate labels assigned by the corresponding isolator or culture collection. The major freshwater cluster is the "Synechococcus cluster" which is represented by ten strains, some isolated from freshwater and some from hot springs. All strains in this cluster contain phycocyanin as their major light-harvesting pigment, phycoerythrin is absent, and all are obligate photoautotrophs incapable of using organic compounds as sole sources of cell carbon.

The first organisms to be discovered by Johnson and Sieburth (1979) and

Table 1.3 Clusters and key feaures of the Synechococcus group (Waterbury & Rippka, 1989)

Cluster	No of strains	cell diameter (μm)	Major light-harvesting pigment	Mol% G+C of DNA	Reference Strain
Cyanobacterium	2	1.7 - 2.3	phycocyanin	39 - 40	PCC 7202 (ATCC 29140)
Synechococcus	10	1.0 - 2.0	phycocyanin	48 - 56	PCC 6301 (ATCC 27144)
Marine A	15	0.6 - 1.7	phycoerythrin	55 - 62	WH 8103 (ATCC 53061)
Marine B	4	0.8 - 1.4	phycocyanin	63 - 69	WH 5701
Marine C	5	1.2 - 2.0	4:phc, 1:phe	47 - 50	PCC 7002 (ATCC 27264)
Cyanobium	8	0.8 - 1.4	phycocyanin	66 - 71	PCC 6307 (ATCC 27147)

Waterbury et al. (1979) were cells with dimensions in the range 0.5 - 1.0 μm x 1.0 - 2.0 μm. Both epifluorescence and transmission electron microscopy revealed these organisms as cyanobacteria. Subsequent studies from oceans have revealed picoplanktonic diatoms, prasinophytes and chlorophycean cells. More recently, with the aid of laser flow cytometry, Chisholm et al. (1988) discovered a picoplanktonic Prochloron. Waterbury et al. (1985) isolated a motile marine cyanobacterial picoplankton. The genus Synechococcus (sensu Rippka et al., 1979) figures most prominently amongst taxonomic identifications made by picoplankton researchers in both marine and freshwater fields, including those of the discoverers of marine cyanobacterial picoplankton (Johnson & Sieburth, 1979; Waterbury et al., 1979). Stockner and Antia (1986) cite records of Cyanodictyon reticulatum, C. imperfectum and Cyanonephron styloides as picoplankton (Hickel, 1981, 1985; Cronberg & Weibull, 1981). However since these colonial organisms are loosely bound by mucilage there must be doubt whether they are true picoplankton.

1.52 Eukaryotic picoplankton

The most commonly reported eukaryote from marine picoplankton was a Chlorella-like coccoid green alga (Johnson & Sieburth, 1982; Joint & Pipe, 1984; Takahashi & Hori, 1984). Two clearly identified Chlorella species of picoplankton size have been reported in the botanical literature; the marine species C. nana was isolated from the Northern Adriatic (Andreoli et al., 1978) and the freshwater C. minutissima was first described in polluted pools near Prague, Czechoslovakia (Fott & Nováková, 1969).

Other eukaryote picoplankters reported include a prasinophycean flagellate

(cell size 1 x 1.5 μm) and a scale-bearing non-flagellated prasinophyte (cell size 0.5 - 1.0 μm) in North Atlantic waters (Johnson & Sieburth, 1982). The latter is believed to be the smallest known autotrophic eukaryote (Stockner & Antia, 1986).

There has only been one report of a diatom in natural size-fractionated marine picoplankton but it was not taxonomically identified (Takahashi & Hori, 1984). There are many reports of small marine diatoms mostly in the nanoplankton range (sensu Sieburth et al., 1978).

Takahashi and Hori (1984) reported a picoplanktonic dinoflagellate in the subsurface chlorophyll maximum layer in subtropical and tropical oceanic waters. Although not identified, a new species is probably involved since there is no picoplankton-sized dinoflagellate in the literature, apart from symbiotic and parasitic forms (Stockner & Antia, 1986).

1.6 AUTOTROPHIC PICOPLANKTON IN THE NATURAL ENVIRONMENT

1.61 Distribution and abundance

Most autotrophic picoplankton research in the last decade has focussed on marine ecosystems and all the oceans (apart from the Black Sea and Red Sea) have data concerning autotrophic picoplankton densities (Stockner & Antia, 1986). Freshwater autotrophic picoplankton reports are fewer and Stockner and Antia (1986) note:

> "... much remains to be done with respect to lakes in Asia, Africa, South America and Australia, which appear from the literature to be virtually uninvestigated".

Published marine field data indicate that in general the relative importance of photosynthetic picoplankton increases from inshore to offshore, from high

latitudes to low latitudes, from winter to summer and from top to bottom of the photic zone (eg Waterbury et al., 1986).

Numerous vertical profiles document Synechococcus distribution and density within water columns (eg Waterbury et al., 1979; Johnson & Sieburth, 1979, 1982; Krempin & Sullivan, 1981; Murphy & Haugen, 1985; Davis et al., 1985; El Hag & Fogg, 1986). Profiles demonstrate that a number of factors contribute to vertical Synechococcus distributions; one of the most important is the depth to which photosynthetically active light penetrates the water column with 1% transmittance, the approximate lower limit for this group of cyanobacteria. In oligotrophic oceanic waters 1% light level occurs at approximately 70 m, but may be considerably shallower in more nutrient rich areas where biomass (particularly algal biomass) attenuates photosynthetically available light.

No studies concerning horizontal autotrophic picoplankton distributions have been reported in the freshwater literature; however, such studies have been carried out in oceans. Waterbury et al. (1986) carried out a 5500 nautical mile north-south transect from the southern tip of South America (53° S) to Woods Hole, Massachusetts (42° N). During the transect between Punta Arenas, Chile and Recife, Brazil in March 1984, Synechococcus densities decreased gradually from a maximum of 3×10^5 cells ml^{-1} at the tip of South America where the temperature was 10 °C to 1×10^3 cells ml^{-1} off Recife where the water temperature was 28 °C. The trend of decreasing cell density with increasing water temperature was contrary to that reported by Murphy and Haugen (1985). In their transect from 37° N to 63° N during April and May 1982, cell densities gradually decreased from 10^5 cells ml^{-1} when water temperature was 18 °C to 10^3 cells ml^{-1} when water temperature was 5 °C.

Waterbury et al. (1986) point to seasonal differences as an explanation for these opposing trends.

1.62 Seasonal variation of autotrophic picoplankton density

There are reports of annual cycles of autotrophic picoplankton density in many ocean provinces. Waterbury et al. (1986) followed Synechococcus abundance in Woods Hole Harbor from 1978 to 1986; this represents the longest study of a single sample station anywhere in the autotrophic picoplankton literature. Synechococcus population density ranged from tens of cells ml^{-1} in winter to a peak of 10^5 cells ml^{-1} in summer. The onset of a spring bloom occurred at the beginning of April when the water temperature reached 6 °C. The bloom was inoculated by the over-wintering population of Synechococcus which ranged in density from 10 to 100 cells ml^{-1}. The bloom period continued through April and May and densities reached a peak of 5 x 10^4 cells ml^{-1} between the middle and end of June when the water temperature was 18 °C. The population density declined following the peak at the end of the bloom period and then varied from 10^4 and 10^5 cells ml^{-1} from July to November.

Freshwater studies (eg Caron et al., 1985; Weisse, 1988; Kennaway & Edwards, 1989) of seasonal autotrophic picoplankton abundance have revealed maximum cell numbers in summer coincident with raised water temperatures and relatively low abundances in winter. Studies by Hawley and Whitton (1988) noted the presence of red-fluorescing and orange-fluorescing autotrophic picoplankton in freshwaters. Kennaway and Edwards (1989) found both types of cells in an upland Welsh lake and reported variable relative abundances throughout the year.

1.7 CULTURING AND ESTIMATING AUTOTROPHIC PICOPLANKTON DENSITIES

1.71 Isolation and culture

The first picoplankter established into culture was the marine, phycoerythrin-rich, clone "Syn 48" isolated by Guillard in 1965. The bloom-forming Synechococcus in Loch Leven was the first freshwater isolate to be cultured from a freshwater environment (Bailey-Watts & Bindloss, 1968).

Pure laboratory cultures are essential for studying many biological aspects of autotrophic picoplankton. In general the strategy for isolating phytoplankton species into culture is to recreate their natural environment as well as possible in the laboratory. Despite these goals, many aims of culturists directly contradict their primary aim. Axenic culture is usually desired and thus species in culture are isolated from the community where they lived previously. The variety of biotic interactions which exist among species may be critically important to the health and survival of some phytoplankton. High biomass is usually desired in laboratory cultures to make various measurements easier and reduce the need for excessively large cultures. Laboratory cultures often have cell densities several orders of magnitude higher than those found in the natural environment. Production of high biomass also requires the addition of high nutrient concentrations to culture media. Autoinhibitory substances that are dilute in natural ecosystems may build up to toxic levels in culture. Thus it is important to realize culture systems are often artifical and may prevent successful isolation of some species in culture.

1.72 Filter fractionation

Measuring autotrophic picoplankton abundance depends on two considerations. First, the organisms of interest must be unambiguously defined. The definition can be based on the stringent application of the actual dimensions of the cells of interest (0.2 - 2.0 μm in width), though a definition based on operational criteria (eg ability to pass through screens of certain pore size) is often more useful in field studies. Second, the property of interest must also be defined (eg biomass, ^{3}H uptake). In freshwater and marine studies autotrophic picoplankton abundance has most often been assessed by measurement of cell number, pigment content or enzymatic activity in aliquots of water passing through selected screens, usually of nominal pore size diameters 1, 2, 3 or 5 μm.

The choice of screen and protocol employed using screens are of paramount importance in determining which organisms can be considered picoplanktonic. Ideal screens would retain all particles of size equal to and greater than its nominal pore diameter. In practice, commonly-used screens (nucleation-track polycarbonate membranes) do not necessarily function ideally. Depending on the filtration protocol, cells smaller than the nominal pore diameter may be retained and larger cells may pass through. Reasons are varied and numerous (Brock, 1983): small cells may be retained on or in screens by clogging, adsorption, electrostatic and Van der Waals forces and impactions. Large cells may pass through by hydraulic shock, cell rupture or may be sufficiently flexible to squeeze through small pores. Thus the use of screens is not necessarily a guarantee that an absolute cut off is achieved.

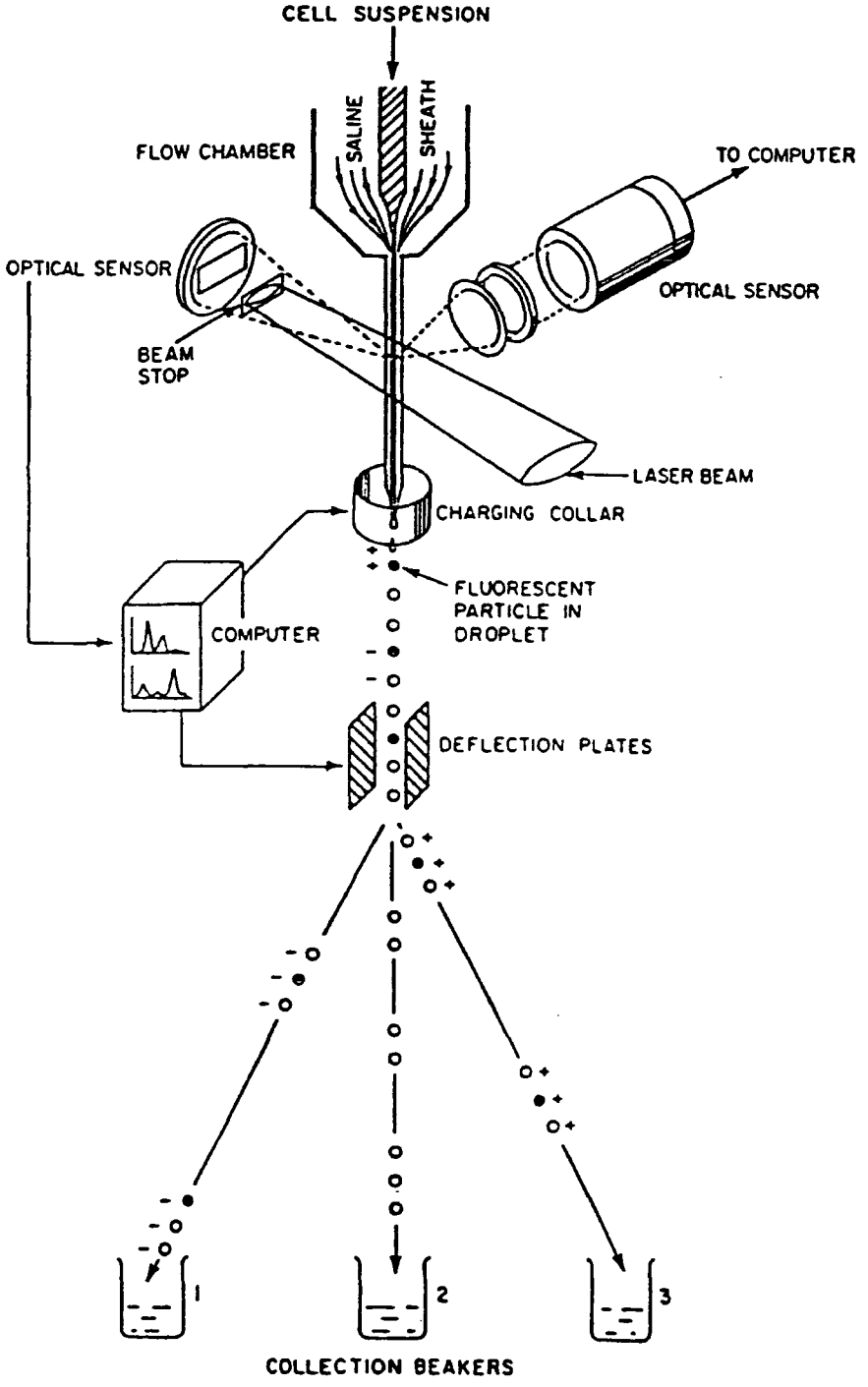
1.73 Detection and enumeration

Microscopic detection and enumeration of photosynthetic picoplankton is aided by the fluorescent emission of visible light when cells are excited by light of shorter wavelength. Algae (and cyanobacteria) belonging to different taxonomic groups possess different pigment suites. Each pigment has different fluorescence excitation and emission maxima. Since pigments occur in particular suites according to taxonomic group, it is possible to attempt rudimentary taxonomic classification from "spectral signatures" or "fingerprints". The most common application of this principle is the enumeration of phycoerythrin-rich cyanobacteria by epifluorescence microscopy and their distinction from algae lacking phycoerythrin whose emitted fluorescence is due mainly to chlorophyll a (Glover, 1985).

In microscopy the human eye detects emitted fluorescence from cells moved about the micrscope stage by human hands. Flow cytometers are an automated method based on the same principle of pigment detection by autofluorescence; electronic photomultiplier tubes quantify relative fluorescence from cells flowing past a beam of excitation light (Fig. 1.3). In addition flow cytometers can provide information concerning the size and granularity of cells based on light scatter. Traditional electronic particle counters eg Coulter Counters resemble fluorescence activated flow cytometers only in that automated analysis is performed on particles flowing past a sensing region. Coulter counters detect particles on the basis of changes to resistivity of a conducting medium as particles flow past charged electrodes. These counters cannot discriminate autotrophic from non-photosynthetic picoplankton.

Detection and enumeration of autotrophic picoplankton can be based on criteria other than the presence of autofluorescent pigments. Some of these

Fig. 1.3 Schematic diagram of a laser flow cytometer with sorting facility (Li, 1987)



include the presence of strain-specific cell surface antigen protein (immunofluorescence) and the ability to incorporate radioactive carbon dioxide by photosynthesis (microautoradiography). In addition, photosynthetic picoplankton are recognizable from electron micrographs of natural assemblages (eg Joint & Pipe, 1984).

Samples collected in the field can rarely be enumerated immediately and they need to be stored in such a way to prevent deterioration. Various preservation methods have been devized including fixing samples in Lugol's iodine for subsequent counts of sedimented organisms (Lund et al., 1958) and fixing samples in formalin or glutaraldehyde. Cells fixed with iodine lose their autofluorescence characteristics and so this method is generally unsuitable for autotrophic picoplankton samples. Cells fixed with formalin or glutaraldehyde retain their fluorescence characteristics and therefore lend themselves to the principal autotrophic picoplankton enumeration methods.

Kuupo-Leinikki and Kuosa (1989) carried out preservation studies on marine autotrophic picoplankton samples and determined optimum storage conditions; both formalin and glutaraldehyde could be used as effective preservatives, but they found freezing without any preservative the best means of storing cells. They found samples could be stored for several months without significant loss in cell counts.

1.8 PICOPLANKTON PHYSIOLOGY

Rates of some physical processes become greatly changed as scale is reduced to picoplankton level. Scale has direct effects on transport and uptake of resources: small cells have increased surface area to volume ratios

for exchange of solutes compared with large cells. Diffusion gradients effectively become steepened around curved surfaces since steady-state flux is inversely proportional to the square of cell radius (Gavis, 1976).

There is also an increase in efficiency of light absorption with decreasing size, an effect which is most marked at wavelengths which are most strongly absorbed. The specific absorption coefficient at the chlorophyll absorption maximum in the red increases some sixfold as cell diameter into which a given concentration of chlorophyll per unit volume of water decreases from 100 to 1 μm (Kirk, 1986).

Small size thus appears to confer selective advantages on phytoplankton in respect to light and nutrient absorption from low concentrations. However diminution below 1 μm diameter confers no appreciable extra advantage and an increase in nutrient flux to cells smaller than this seems to be in excess of anything needed to support even the most rapidly growing organisms. Raven (1986), following Pirie (1964), calculated that minimum cell size is set by minimum genome size and the number of catalysts required to maintain life; for a photosynthetic organism this is probably a 0.3-μm diameter sphere. It therefore seems that the lower size limit of 0.2 μm used to define picoplankton is in fact near the limit at which these organisms are theoretically possible. The upper picoplankton size limit is more arbitrary and the advantages of small size extend well into the nanoplankton range.

Cyanobacteria generally attain maximum growth rates at relatively low irradiances and picoplankton representatives share this trend. Those which have been studied in culture show low compensation points (the point at which carbon fixation from photosynthesis is the same as carbon burnt in

respiration) and maximum relative growth rates of between 0.5 and 2 doublings per day at 20 - 25 °C and around 50 μmol photon m^{-2} s^{-1}; photosynthesis saturates at 100 μmol photon m^{-2} s^{-1}.

Picoplanktonic cyanobacteria contain pigments which allow efficient use of the wavelengths most available in the sea. Strains isolated from inshore stations have a predominance of blue phycocyanin whereas those from oceanic waters contain more phycoerythrin and have an absorption maximum in the green. From flow cytometry data, Olson et al. (1985) found a Synechococcus population with a fourfold brighter phycoerythrin fluorescence in deeper water than those higher in the water column in the region of the thermocline in the Gulf stream off the USA. In other cyanobacteria, light energy absorbed by this pigment is transferred with high effeciency to reaction-centre chlorophyll (Duysens, 1952); Wood (1985) has shown that strains containing phycoerythrin photosynthesised more effectively at 60 m depth than a strain without it. However a phycoerythrin-containing Synechococcus strain found by Barlow and Alberte (1985) had a high in vivo fluorescence yield from phycoerythrin, indicating that it is not part of the photosynthetic system. They suggested phycoerythrin, which may make up as much as half total cell protein, acts as a nitrogen reservoir. It has been confirmed that Synechococcus grown with ample combined nitrogen has a high fluorescence yield when illuminated with green light and these cells sustain high growth rates for one or two days when deprived of a nitrogen supply (Wyman et al., 1985; Alberte & Kirchman, 1985). The nitrogen deficient cells produced by this treatment show lower phycoerythrin content and low fluorescence yield, consistent with its participation in photosynthesis. Apart from acting in this way as an available nitrogen reserve it is possible

that phycoerythrin may have a protective role against damaging effects of high irradiance.

Waterbury et al. (1986) examined 49 strains of unialgal or axenic marine Synechococcus strains for a variety of physiological parameters, including motility, heterotrophic growth, chromatic adaptation and growth in different nitrogen sources. Motility was observed in five strains and consisted of a novel swimming method for which a plausible mechanism is presently lacking (Waterbury et al., 1985). Many filamentous and some unicellular cyanobacteria are capable of gliding motility when in contact with solid surfaces (Castenholz, 1982) but motile Synechococcus swam in open water with bacterial-like motion though no evidence for flagella was found.

Most cyanobacteria are photoautotrophs, but many have the ability to grow photoheterotrophically in the light and some are capable of facultative chemoheterotrophic growth in the dark (Rippka, 1972; Rippka et al., 1979). Marine strains tested by Waterbury et al. (1986) all appeared to be obligate autotrophs, though this does not preclude the possibility that they are able to assimilate a variety of organic compounds into some cell constituents. All strains tested by Waterbury et al. (1986) were capable of using nitrate and ammonia as the sole source of nitrogen and about half could utilize urea. They tested eighteen axenic strains for their ability to induce nitrogenase activity but all were negative.

Some cyanobacteria are capable of adapting chromatically - the process by which different accessory pigments are synthesized depending on the light quality in the environment. None of the strains tested by Waterbury et al. (1986) exhibited chromatic adaptation, the relative rates of synthesis of phycoerythrin and phycocyanin were constant for a given strain and unaffected

by light quality.

Synechococcus strains maintained by Waterbury et al. (1986) grew optimally between 20 - 25 °C. All isolates were isolated from oceans in temperate latitudes and none grew at temperatures in excess of 30 °C.

1.9 PICOPLANKTON ECOLOGY

1.91 Grazers

Organisms may be expected to feed on particles one order of magnitude smaller in size than themselves (Azam et al., 1983) and accordingly it is found that most mesozooplankton do not feed on picoplankton (Joint & Morris 1982; Azam et al., 1983; Jorgensen, 1984). Although picoplanktonic cyanobacteria have been found in gut contents and faeces of Calanus finmarchicus, they are not digested and persist intact (Johnson et al., 1982) as they do also in freshwater copepods (Caron et al., 1985). The freshwater cladoceran Daphnia cucullata however, is able to take substantial part of its food requirement in the form of bacteria (Riemann & Bosselman, 1984).

Size considerations suggest that picoplankton grazers will be amongst nanozooplankton (2 - 20 μm) and the importance of these was recognized by Lohmann (1911). It is difficult to distinguish between phototrophic and phagotrophic flagellates in this size class (Sieburth & Davis, 1982) which includes choanoflagellates, cryptophytes, dinoflagellates, chrysomonads, bodonids and bicoecids (Fenchel, 1982 a,b; Sherr & Sherr, 1984). These are almost exclusively phagotrophic on bacteria and phototrophic picoplankton (Taylor, 1982). There are positive correlations between numbers of flagellates in this size and bacteria with which they occur in approximately

1:1000 ratio, flagellate numbers being nearly always around 10^3 ml^{-1} in both oligotrophic and eutrophic waters (Sieburth & Davis, 1982; Fenchel, 1982 a,b; Sherr & Sherr, 1984; Davis et al., 1985; Gast, 1985). Transmission electron micrographs provide evidence of picoplankton ingestion (Sieburth & Davies, 1982). In Lake Ontario samples up to 15 % of the microflagellate population contained ingested coccoid cyanobacteria when the density of the latter exceeded 10^5 cells ml^{-1} and it was concluded that flagellates are important consumers of cyanobacteria (Caron et al., 1985).

Fuhrman and McManus (1984) obtained evidence suggesting that organisms rather smaller than flagellates may be important in grazing bacteria. By using inhibitors specific to eukaryotic metabolism with filtered sea water samples, they obtained evidence suggesting that much bacterial grazing was attributable to eukaryotic organisms less than 0.6 μm diameter. Ciliates are said to be poorly adapted to capture particles less than 1 or 2 μm diameter (Conover, 1982; Fenchel, 1984). In freshwaters ciliates appear able to subsist on freely suspended bacteria in the densities usually present (Sieburth, 1984) but it seems doubtful whether most marine species share this ability (Sieburth, 1984).

1.92 Role in planktonic food chains

Organic matter contributed by autotrophic picoplankton primary production appears to be recycled almost entirely within the microlitresphere if the water is oligotrophic and larger organisms scarce. Autotrophic picoplankton is not grazed appreciably by mesoplankton, but together with bacterioplankton, which derives its carbon from dissolved organic matter, are consumed by phagotrophic flagellates, which in turn are consumed by ciliates.

It is not clear to what extent this "microbial loop" (Azam et al., 1983) may contribute to secondary production of larger organisms in classical planktonic food chains. Ciliates are themselves preyed on by a wide variety of larger zooplankton and thus may form a link between picoplankton and larger secondary producers (Conover, 1982). Williams (1984) suggested 16% of bacterial production may reach mesozooplankton.

The contribution of the microbial loop to pelagic ecosystems may be much greater in mineral cycling terms. Picoplankton is particularly efficient at taking up dissolved organic matter so excretions and soluble matter from dead organisms, both large and small, are likely to be used mainly at this level. Williams (1981) concluded at least half the primary production must pass through microheterotrophs before it is mineralised; Taylor (1982) put forward general arguments for believing bacteria and phagotrophic flagellates help maintain high production rates by facilitating nutrient regeneration. Photosynthetic picoplankton would be expected to benefit most from mineralization as a result of their high uptake capacity and their intimate association with bacteria and flagellates.

1.10 AIMS OF PRESENT WORK

1. To standardise and improve methods for sampling, preserving and counting autotrophic picoplankton.
2. To make a broad survey to establish the importance of autotrophic picoplankton in freshwaters and establish key features of their biology in laboratory studies.

3 stages in the research programme were identified to fulfil the aims:

* Establish a methodology which would be used throughout the project.
* Make a broad survey of autotrophic picoplankton in freshwaters.
* Isolate strains in culture and carry out laboratory studies.

CHAPTER 2

METHODS

2.1 SAMPLING

Data are included from a range of sites including standing water bodies and flowing waters. Samples taken as part of a routine sampling programme in the UK (Chapter 5) were taken from a depth of 0.5 m. Where possible a boat was used to take the sample from a position over the deepest point in the basin; when this was not possible samples were taken at a distance from the edge where the water was 1 m deep or greater. Samples were collected in a Ruttner bottle. Samples taken from flowing waters were collected in a polythene bottle just below the surface. Colleagues and expeditions collecting samples from overseas were instructed to collect samples from the middle of the lake if possible, but in practice most were collected from the lake shore.

2.2 AUTOTROPHIC PICOPLANKTON ENUMERATION

2.21 Prefiltration

Samples from nutrient rich environments needed to be prefiltered to remove larger phytoplankton cells whose bright fluorescence masked the fluorescence from autotrophic picoplankton cells. A 3-μm filter was chosen as Craig (1983) suggests smaller pore sizes (ie 2-μm filters) may retain picoplankton at the upper end of the range. Although 3-μm screens allowed some meso-plankton cells through (sensu Sieburth et al., 1978), measurement with a

graticule eye-piece excluded these cells from autotrophic picoplankton counts.

2.22 Enumeration

Samples were prefiltered under gravity through a 3.0 μm Nuclepore polycarbonate membrane and then preserved with buffered formalin (pH 7.0, 2% final concentration). Between 5 and 50 ml (depending on autotrophic picoplankton density in the sample) was drawn through a 25 mm diameter 0.2 μm Nuclepore filter under partial vacuum (< 100 mm Hg) that had been prestained for twenty minutes with irgalan black (Ceiba-Geigy, 2 g l^{-1} in 2% acetic acid). A glass fibre filter (Whatman GF/C) was placed between the polycarbonate membrane and the filter holder as a backing filter to create an even vacuum and so draw cells randomly onto the membrane. A goodness-of-fit χ^2 test for randomness was carried out on fifty preparations. Filters were mounted on glass slides that had been pre-chilled at 4 °C for twenty minutes; the condensation formed on the slide when returned to room temperature ensured the filter attached firmly to the slide, without creasing. A drop of non-fluorescent immersion oil (Cargille type A) was placed on filters and a coverslip gently placed on top, sandwiching the oil evenly between filter and coverslip. Uneven pressure at this stage caused cells to move non-randomly on the filter. Preparations were enumerated under epifluorescence microscopy (Nikon Fluophot) using a x 100 oil immersion objective. Autofluorescent cells in the picoplankton size range (measured by micrometer eye-piece) were detected by green (ca. 500-550 nm) and blue (ca. 420-490 nm) excitation filter sets. A total of 400 cells in fields of view were counted to give a counting precision of 10% (Lund et al., 1958) and the mean

number of cells per field calculated.

2.23 Calculation

The multiplication factor equating the area of the field of view to the cross-sectional area of the filter was calculated by measuring the inside diameter of the filtration chimney to obtain its cross-sectional area and measuring the size of a field of view in the microscope with a stage micrometer to determine its area. The number of cells per ml was then calculated from:

$$\frac{\text{cells field}^{-1}}{\text{ml sample filtered}} \times (1.02 \times 10^4)$$

where cells field^{-1} was the mean number of cells in a field of view
1.02×10^4 was the multiplication factor

2.24 Biovolume

Twenty five cells on each filter were randomly selected and measured to the nearest 0.25 μm using a calibrated micrometer eye-piece. Cell biovolume was calculated from geometric shapes corresponding to cell shape. In all samples examined autotrophic picoplankton were either short rods or coccoid shaped. The biovolumes of rods were calculated by assuming cells were a combination of a cylinder and two hemispheres; the biovolume of coccoid cells was estimated by calculating the volume of a sphere with radius equal to the radius of the cell.

2.3 ENVIRONMENTAL VARIABLES

2.31 Physical variables

Dissolved oxygen, temperature and conductivity were measured using WTW (Wissenschaftlich Technische Werkstätten) meters (model 191) and probes. pH was measured using an Ingold Xerolyte probe coupled to a WTW 191 pH meter. Oxygen and pH meters were calibrated prior to each reading according to the manafacturer's instructions.

2.32 Chlorophyll *a*

Aliquots of lake water (1 l in meso- or eutrophic lakes, 3-5 l in oligotrophic lakes) were filtered under vacuum through a 47 mm Whatman GF/F glass fibre filter (nominal retention size 0.7 μm) using a Nalgene vacuum filter holder and hand vacuum pump. The filter was placed in 5 ml of 90% methanol in a 30 ml universal bottle and stored at 4 °C in the dark for 12 h to extract the pigment (Carr & Goulder, 1990). Extracts were then filtered through a GF/C glass fibre paper to remove cell debris. The optical density of the resultant pigment extract was measured at 665 nm against a solvent blank; subtraction of the optical density at 750 nm from this value compensated for sample turbidity. Extracts were then acidified by the addition of 5 μl of 1.0 N HCl. Acidified extracts were left for 1 h at room temperature (and then neutralised) before absorbances at 665 nm and 750 nm were re-read. Chlorophyll *a* was calculated from:

$$\text{Chl } a \ (\mu g/\text{litre}) = 2.61 \ (a_b - a_a) \times V/L \times 13.1$$

where a_b = absorbance at 665 nm - absorbance at 750 nm before acidification

a_a = absorbance at 665 nm - absorbance at 750 nm after acidification

13.1= constant, assumes a specific absorption coefficient of chl *a* in 90% methanol of 76.07 g l^{-1} cm^{-1}

2.61 = constant derived from an acid factor.

V = volume of methanol in which pigment is extracted

L = path length of the cuvette

2.33 Phosphorus

Phosphorus was measured according to the method of Eisenreich *et al.* (1975). Samples from lakes were collected from 0.5 m depth in iodised polyethylene sample bottles and transferred to the laboratory at 4 °C in the dark; analysis was completed within 12 h of sampling. A 10 cm cuvette was used in each case and the detection limit of the system was 2 μg P l^{-1}. For each of the water samples the total filtrable phosphate fraction was analysed; this involved filtering the raw water sample through a GF/F filter and autoclaving the filtrate with acid to digest organically bound phosphorus (eg nucleic acids). All the free phosphate ions were then quantitatively complexed with molybdate to form a blue complex which was measured spectrophotometrically.

2.4 COMMON LABORATORY PROCEDURES

2.41 pH

All laboratory pH measurements were carried out with an Ingold combination electrode and EIL pH meter (model 7050). The probe was calibrated with BDH standard buffer solutions. Measurements could be made with a precision of $\pm$0.05 of a pH unit.

2.42 Light

Light measurements were made with a Macam light meter (model Q101). All incident light was measured as photosynthetically active radiation (PAR) and readings were recorded as photon flux density (μmol photon m^{-2} s^{-1}). In culture experiments where light intensity was varied (Section 7.3) neutral density filters were wrapped around culture vessels to reduce incident light to the required intensity.

2.43 Absorption

A Shimadzu Digital Double-Beam Spectrophotometer (model UV-150-Z) with glass cuvettes (4 cm or 10 cm) was used for single wavelength readings.

2.5 CULTURE TECHNIQUES

2.51 Durham Culture Collection

All unialgal strains in the collection are assigned a unique 3-digit number. The culture records are held on a Research Machines Nimbus X16 operating under MS-DOS; the software used was Superfile (Southdata, London).

2.52 Origin of cultures

A list of the strains used in the study is presented in Table 2.4 along with their country of origin and environments from where they were isolated. 9 strains were rendered axenic by the author and of these, 8 (D767, D769, D773, D774, D798, D799, D807, D838) were isolated by the author from water samples collected in the field by himself or colleagues.

Table 2.1 Axenic autotrophic picoplankton strains used in laboratory studies.

Durham Culture No.	Organism	Country found	Date	Site details	Axenic on	Cleaned by	Cleaning method
D033*	Anacystis nidulans	U.S.A.	ca. 1952	Stream	ca. 1955	W.A. Kratz	unknown
D767	Synechococcus	Bangladesh	12/10/85	DWR field	23/02/87	G.R.W. Hawley	aerosol
D548	Chlorella	NA	ca. 1933	NA	NA	NA	NA
D562	Synechococcus	U.S.A.	22/08/79	Elvins Tailings	20/08/87	P.P. Jackson	repeated streaking
D768	Synechococcus	Bangladesh	12/10/85	Open area, DWR	22/11/85	J.W. Simon	aerosol
D769	Synechococcus	Bangladesh	12/10/85	Open area, DWR	03/07/87	G.R.W. Hawley	aerosol
D772	Synechococcus	U.K.	22/10/85	Greenhouse tank	11/04/87	G.R.W. Hawley	aerosol
D773	Synechococcus	U.K.	22/10/85	Greenhouse tank	05/03/87	G.R.W. Hawley	aerosol
D774	Synechococcus	U.K.	22/10/85	Cassop Pond	05/04/89	G.R.W. Hawley	flow cytometry
D797	Synechococcus	Nepal	18/09/86	Kakaoni rice field	01/06/87	J.W. Simon	aerosol
D798	Synechococcus	Bangladesh	13/09/86	Sonargaon DWR field	05/04/89	G.R.W. Hawley	flow cytometry
D799	Synechococcus	Bangladesh	13/09/86	Dhaka park lake	05/04/89	G.R.W. Hawley	flow cytometry
D807	Synechococcus	Bangladesh	13/09/86	Sonargaon DWR field	25/06/87	G.R.W. Hawley	aerosol
D810**	Synechococcus	International	NA	Atlantic ocean	NA	NA	NA
D838	Synechococcus	U.S.A.	23/04/87	Mountain Lake	10/06/89	G.R.W. Hawley	flow cytometry

NA = no data available

* This organism corresponds to the following strains in other culture collections: CCAP 1405/1, SAUG 1402/1, UTEX 625, ATCC 27144. The strain may be known by its synonym Synechococcus leopoliensis (Racib.) Komárek.

** The strain is synonymous with DC2, CCMP WH7803, SMBA 314.

2.53 Preparation of media for batch culture

To prepare 1 litre of medium, 400 ml of glass-distilled water was buffered with 0.6 g HEPES. This buffer was chosen because of its reported lack of interference with biological systems (Smith & Foy, 1974). The addition of HEPES lowered the pH to ca. 5.0 and this was adjusted to pH 7.0 by the dropwise addition of 1.0 M NaOH. The buffered solution was transferred to a 1-litre volumetric flask into which media mineral salts were added in a specific order. Upon addition of each salt the flask was swirled gently to mix the salts and prevent any reaction as a result of unusually high local concentrations. After the addition of all the salts the final volume was adjusted to 1 litre with glass-distilled water. Medium was made up as required from stock solutions stored in the dark at 4 °C. 25 ml aliquots of medium were dispensed into 100-ml conical flasks; these were plugged with silicon bungs (type S28, SANKO Plastic Co Ltd., Japan) and autoclaved.

2.531 Chu 10-N NH_4N (10) medium

This medium was selected as it is a relatively dilute inorganic medium. Aziz and Whitton (1988) judged Chu 10 medium to be the closest medium to water in Bangladesh rice fields at the time of flooding; as six strains were isolated from this environment it was decided to use this medium and not the more commonly used medium BG-11. Also, BG-11 was avoided in these studies as it contains an organic source of iron and very high phosphate levels which were thought to be unrepresenative of environments from which any of the strains had been isolated. Mineral salt composition of the medium is presented in Table 2.2.

2.532 Artificial seawater medium.

Artificial seawater medium was based on the recipe given by Wyman and Carr (1985). The recipe was modified by omitting organic iron and replacing it by an iron EDTA mixture at the same concentration as in the freshwater medium. Mineral salt composition of the medium is given in Table 2.3. The same microelement mixture was used for both marine and freshwater media; its chemical composition is given in Table 2.4.

2.54 Sterilization

All flasks and tubes containing medium were sterilized at 121 °C (10.35 KN m^{-2}; 15 lb in^{-2}) for 15 min. Media were allowed to stand for at least six hours (generally overnight) before inoculation to allow requilibration with the atmosphere.

2.55 Culture vessels

100-ml pyrex Erlenmeyer flasks were used for the maintenance of stock cultures. Experiments were conducted in 50 ml boiling tubes with Morton closures.

2.56 Cleaning

All glassware was soaked in 2% Decon-90, a phosphate-free detergent, for 1 h and then rinsed six times in glass-distilled water. Clean glassware was dried at 105 °C before use.

Table 2.2 Salt composition of Chu 10-N NH_4-N (10) medium. Medium was buffered with 25 mM HEPES and adjusted to pH 7.0 with 1.0 M NaOH

Compound/ element	Molecular mass	Stock conc^n (g l^{-1})	medium conc^n (mg l^{-1})	(mM)
NH_4Cl	53.49	3.82	38.2	0.71
N	14.007		10	
Cl	35.453		25.3	
$KH_2PO4.3H_2O$	136.090	15.6	7.8	0.06
K	39.098		2.24	
P	30.974		1.78	
$CaCl_2.2H_2O$	147.02	35.83	35.83	0.24
Ca	40.080		9.76	
Cl	35.453		17.31	
$MgSO_4.7H_2O$	246.470	25.0	25.0	0.10
Mg	24.305		2.47	
S	32.060		3.25	
$NaHCO_3$	84.010	15.85	15.85	0.19
Na	22.989		4.32	
C	12.011		2.26	
Na_2EDTA	372.240	13.35	3.34	0.009
Na	22.989			
EDTA	326.262		0.44	
$FeCl_3.6H_2O$	270.300	9.700	2.43	0.009
Fe	55.847		0.07	
Cl	35.453			
Buffering				
NaOH	40.000	40.000	34.00	0.85
Na	22.989		19.54	

Table 2.3 Salt composition of artificial seawater medium, based on the recipe used in Wyman and Carr (1985). The medium was buffered with 1.1 g l^{-1} Tris and the pH was adjusted to 8.0 with 1 M HCl

Compound / element	Molecular mass	Medium conc^{n} ($g\ l^{-1}$)	(mM)
NaCl	58.44	25	0.43
Na	22.9898	9.83	
Cl	35.435	15.15	
$MgCl_2.6H_2O$	203.30	2	0.01
Mg	24.305	0.24	
Cl	35.453	0.70	
KCl	74.55	0.5	0.007
K	39.0983	0.26	
Cl	35.453	0.24	
$NaNO_3$	84.99	0.75	0.009
Na	22.9898	0.2	
N	14.0067	0.12	
$K_2HPO_4.3H_2O$	174.18	0.03	0.0001
K	39.0983	0.013	
P	30.9738	0.005	
$MgSO_4.7H_2O$	246.47	3.5	0.01
Mg	24.306	0.345	
S	32.06	0.46	
$CaCl_2.2H_2O$	147.02	0.5	0.003
Ca	40.08	0.14	
Cl	35.453	0.24	

Table 2.4. Salt composition of microelements used in both freshwater and marine media

Compound/ element	Molecular mass	Stock conc^{n} (g l^{-1})	medium conc^{n} (mg l^{-1})	(mM)	total element (mg l^{-1})	(mM)
H_3BO_3	61.830	2.86	0.7150	0.0116		
B	10.810		0.1250		0.1250	0.0116
$MnCl_2.4H_2O$	197.920	0.181	0.0453	0.0002		
Mn	54.938		0.0126		0.0126	0.0002
Cl	35.453		0.0162		0.0162	0.0005
$ZnSO_4.7H_2O$	287.550	0.222	0.0555	0.0002		
Zn	65.380		0.0126		0.0126	0.0002
S	32.060		0.0062		0.0099	0.0003
$CuSO_4.5H_2O$	249.680	0.079	0.0198	0.00008		
Cu	63.546		0.0050		0.0050	0.00008
S	32.060		0.0025			
$CoSO_4.7H_2O$	281.100	0.042	0.0105	0.00004		
Co	58.933		0.0022		0.0022	0.00004
S	32.060		0.0012			
$Na_2MoO_4.2H_2O$	241.950	0.027	0.0068	0.00003		
Na	22.989		0.0013		0.0013	0.00006
Mo	95.940		0.0027		0.0027	0.00003

2.57 Aseptic technique

Inoculation of flasks at the beginning of experiments, and for culture maintenance, was carried out using standard aseptic techniques in a vertical laminar flow hood (Microflow Pathfinder).

As part of the method to render strains axenic, cultures were sprayed (Section 2.821) on to solid media. Colonies which grew subsequently on the media were then transferred into fresh liquid media. This transfer was carried out using sterile micropipettes which were made by pulling pasteur pipettes having first softened them in a bunsen burner. The micropipettes were sterilised before use. Colonies of Synechococcus were easier to pick up from agar with these micropipettes compared with an ordinary microbiological loop.

2.58 Culture purity

The purity of axenic strains was tested before each culture experiment by plating inoculum on 1% (w/v) agar plates supplemented with the following media:

1) beef peptone
2) malt extract
3) yeast extract
4) nutrient broth
5) SST

Composition of these media are described by Hoshaw and Rosowski (1973). In addition agar plates were made up with the usual growth medium supplemented with 0.02% casamino acids (Bacto-Difco) and 2% glucose. Plates were incubated in the dark at 32 °C for three days and then examined. Bacterial growth was usually well developed by this time in contaminated cultures.

2.59 Incubation and light source

Experiments were conducted in either a constant temperature growth room or a thermostatically-controlled water tank with shaking mechanism. Vessels in growth rooms were shaken by hand twice daily. In tanks, culture vessels were illuminated continuously from below with warm white fluorescent tubes. The photon flux density at the vessel surface was variable with location, particularly at the edges of the tank. Vessels were incubated in a wire rack in the central portion of the tank to overcome this, and within this area vessels were moved randomly each day. The light source in the growth rooms was from warm white fluorescent tubes located above vessels.

2.60 Estimating population density

Cell counts were estimated using a Hawksley 0.1 mm haemacytometer with improved Neubauer rulings. Cells were left to settle for five minutes after loading the chamber so that most were in a single plane of focus thus aiding counting. Cultures generally maintained discrete unicells but in some cases old cultures tended to clump together; when this happened, samples were sonicated for 30 s to break up clumps prior to counting.

2.61 Inoculum

Inoculum for experiments was taken from log phase cultures grown at 32 °C. Cell density was adjusted so that experimental vessels contained a cell density of 10^5 cells ml^{-1} at the beginning of the experiment. 10^5 cells ml^{-1} was chosen as this represented the detection limit of the 0.1 mm haemacytometer.

2.62 Doubling time

Doubling times (t_d) from growth curves were calculated from the steepest region of the curve (assessed by eye). Three points in this part of the curve were chosen and the regression was computed of the Napierian logarithm (ln) of these cell densities with time. The slope of this line is the growth constant, K_e, and the number of divisions per day (k) is given by $K_e/\ln 2$. From k, the doubling time or generation time td was:

t_d = 1/k days per division = 24/k hours per division

2.63 Coefficient of variation

The coefficient of variation (CV) was used to compare the relative variability of samples. It is the term applied to the standard deviation when it is expressed as a percentage of the sample mean. It was calculated from the formula:

CV = s.(100/x) where x = sample mean and s = standard deviation of the sample.

2.7 PHYSIOLOGICAL METHODS

2.71 Alkaline phosphatase

Alkaline phosphatase is an enzyme which cleaves the phosphate molecule from a complex organic molecule; the phosphate molecule therefore becomes part of the pool of soluble reactive phosphorus and can be taken up readily by autotrophic cells. The ability of a cell to produce the enzyme allows it to use a source of phosphorus which would otherwise be unavailable. There are two main main phosphatase enzymes, a phosphomonesterase which cleaves a phosphate molecule from an organic molecule and a phosphodiesterase enzyme

which cleaves two phosphate molecules from an organic molecule. In the alkaline phosphatase assay the organic molecule (substrate)is a colourless molecule, p-nitrophenyl phosphate (for assaying phosphomonoesterase activity) and bis p-nitrophenyl phosphate (for assaying phosphodiesterase activity). The enzymes cleave the phosphate from the substrate leaving p-nitrophenyl (pNP) and bis-p-nitrophenyl (bis-pNP) both of which are coloured yellow. The assay detects the development of a yellow colour which is measured spectrophotometrically.

2.711 Assay method

Cultures were centrifuged in 50 ml MSE non-sealable polyethylene centrifuge tubes in a SS-34 8 x 100 ml angle head rotor using a Sorvall RC-5B refrigerated superspeed centrifuge at 8000 x g for 20 min. The supernatant was decanted and filtered through a GF/C filter (Whatman) and was used to determine the extracellular phosphatase fraction. The term cell bound phosphatase is used to indicate the location of the activity in centrifuged cells.

The algal pellet was washed twice in de-ionised water and resuspended in assay medium which was 1.4 times its normal concentration; this allowed for the dilution effect in the assay.

For cell-bound and extracellular alkaline phosphatase activity (APA) a 30-μl aliquot of algal sample, or supernatant, was pipetted via a Titertek 8-channel pipettman (EFLAB, Finland) into a 2% Decon-washed 96 microwell plate (no. 96F, Inter Med, NUNC, Denmark). Each sample was replicated eight times. All microwell plates had lids to reduce contamination and reduce evaporation; lids also acted as insulators against heat-loss. 90 μl of

buffer [glycine-NaoH, pH 10.3 (50 mM final concentration)]was pipetted in the microwells. This buffer was chosen rather than AMP which is the recommended buffer in the Sigma method as the activity was higher in glycine-NaOH. Grainger (1989) tested the pH optima of the phosphatase enzyme for a number of strains and found activity was generally greatest at pH 10.3. Preliminary experiments using Synechococcus strains indicated that maxima were at this pH also. Assay plates were incubated at 32 °C for 30 min before the addition of the substrate to allow the solutions to reach assay temperature. 180 μl of 0.25 mM pNPP or 0.5 mM bis-pNPP (7.75 mg l^{-1} P final concentration) was then pipetted into microwells and this marked the beginning of the assay. A T=0 min reading was taken and subsequent readings at 10 min intervals for 30 min. Subsequently a time course was plotted and a value for APA was taken from the linear part of the curve. A calibration curve was constructed using p-nitrophenol (pNP) between 0.002 - 0.2 μmol. Activity was expressed as μmol pNP mg d.wt^{-1} h^{-1}.

2.712 MCC plate reader

An MCC plate reader was used for colorimetric analysis of alkaline phosphatase activity (APA). Absorbance Program 1 and Filter Code 1 were used, which measured the absorbance of the assay at 405 nm.

2.721 Acetylene Reduction Assay

The acetylene reduction assay was used for estimating nitrogenase activity in strains. Strains were grown in Erlenmeyer flasks to nitrogen limitation (12 days). Bungs were then removed and replaced by suba-seals. 10 ml of air was removed from the incubation vessel and 10% C_2H_2 v/v added using a 20

ml syringe (Beckton-Dickinson, Ireland). The gas phase was allowed to equilibrate to atmospheric and each vessel gently swirled to ensure homogenous gas phase. Incubation time was 1 h. Following incubation, the flasks were again swirled before the removal of 1 ml of gas with a 1-ml disposable plastic syringe fitted with a 0.5 mm needle (Beckton-Dickinson, Ireland). Tips were placed immediately in rubber bungs (previously tested to ensure no ethylene release). Samples were subsequently injected into a Varian Aerograph (model 1400) gas chromatograph fitted with a flame ionization detector. Samples were run against ethylene as well as ethylene and acetylene standards covering working ranges.

2.722 Oxic, micro-oxic and anoxic environments.

Acetylene reduction assays were conducted in a range of oxygen partial pressures. Oxic conditions refer to cultures grown in Erlenmeyer flasks in atmospheric oxygen. Micro-oxic conditions were created by flushing the flasks with argon and allowing the atmosphere to re-equilibrate before re-introducing 10% v/v air. This treatment resulted in an O_2 partial pressure ca 2% atmospheric. Anoxic conditions were created by flushing flasks with argon.

2.73 Heterotrophic growth

Glucose, sucrose, fructose, galactose, maltose and acetate were added to standard growth medium at a concentration of 0.01 M after filter sterilization through a Nuclepore 0.2-μm membrane. Flasks were inoculated with autotrophic picoplankton strains and incubated in the dark at 32 °C for 6 weeks. After this time, aliquots were removed and loaded into a

haemacytomter to estimate cell density and hence assess growth.

2.74 Cryo-preservation

All clonal cultures, whether axenic or unialgal, were stored under liquid nitrogen at -184 °C.

2.75 Desiccation tolerance

Some isolates of blue-green algae from a number of habitats, including rice fields, are desiccation tolerant (Whitton, 1987); they survive the dry season protected by mucilage and following re-wetting in the next rainy-season become metabolically active once again. Since a number of strains isolated in this study originated from a similar environment (D767, D768, D769, D797, D798, D799, D807), experiments were conducted to determine their desiccation tolerance.

Sterile strips of cotton gauze or "Agrosoke" were placed in exponentially growing cultures for 1 h. Strips were then removed and placed in sterile petri-dishes where they dried out. Dried strips were re-wetted one week later in fresh medium. Cultures were examined microscopically for signs of growth.

2.76 Motility

A number of motile strains of marine autotrophic picoplankton have been isolated (Section 1.51) but there are no reports of any equivalent cells from freshwater environments. The presence or absence of motile cells in strains isolated in this study was assessed microscopically. Aliquots of cultured material from both exponential and stationary phase material was placed on

cavity slide and viewed under a Nikon Fluophot microscope using a x 40 objective.

2.8 ISOLATING SRAINS

A number of different methods were used in attempts to render strains axenic. In all cases where strains were isolated from "raw' water samples, an enrichment culture was made first. After obtaining a culture from the enrichment process, it was plated out on agar using the aerosol method (see below); this was to permit single cells to grow into colonies which could be picked up and transferred to liquid media. This ensured that strains that were rendered axenic would be clonal.

2.81 Enrichment culture

Sample water was prefiltered through a 3.0-μm Nuclepore membrane into a flask of Chu 10-N NH_4N medium. Final medium concentration was quarter strength. Flasks were incubated at 32 °C in 100 μmol photon m^{-2} s^{-1}. Enrichment cultures were inspected weekly for signs of growth. Cultures with visible signs of growth (a green, or blue-green tinge to the medium) were subcultured in full strength medium. There was no picoplanktonic eukaryotic growth in any of the enrichment cultures. This may have been because cyanobacterial picoplankton were far more numerous, or because the culture conditions favoured cyanobacteria.

2.82 Rendering strains axenic

Cultures were cleaned by one or combinations of different methods. In all the methods aliquots of exponentially growing cultures were chosen for

cleaning. This was because the autotrophic to heterotrophic ratio of cell number was likely to be greatest. Old cultures often produced mucilage and this may have encouraged the growth of bacterial cells.

2.821 Aerosol

1 ml of a logarithmically growing culture was serially diluted into four tubes of sterile media. 100 μl from each of the tubes was dropped into a stream of sterile compressed air; the drop became an aerosol and droplets were blown onto an agar plate. Five aliquots from each dilution were collected on plates which were subsequently incubated at 32 °C in 100 μmol photon m^{-2} s^{-1} for 3 - 4 weeks. The plates were examined under a stereo microscope and the plates with colonies with the least contamination were selected for further treatment; the rest were discarded. Generally, just one of the sets of plates from the original serial dilution series yielded a number of colonies which appeared to be bacteria-free. The other dilutions yielded plates in which all colonies were contaminated or occassionly no colonies grew at all. Colonies which were apparently free from bacterial contamination (contaminated colonies had a "halo" of bacteria) were picked off aseptically using sterile micropipettes and resuspended in fresh medium in Durham tubes. Typically, about a dozen colonies were isolated in this way from a plate and they were incubated in the Durham tubes at 32 °C and 100 μmol photon m^{-2} s^{-1} until the medium had a blue-green tinge (approximately 10^5 cells ml^{-1}). Aliquots of these cultures were tested for bacterial contamination by steaking on supplemented agar plates (Section 2.58). In the event that none of the tubes contained a culture of axenic cyanobacteria the process was repeated using the culture which was least contaminated

(judged by the amount of bacterial growth on the test plates). Repetition of the process three or four times resulted in axenic cultures of seven strains (Table 2.1).

2.822 Dilution

Logarithmically-growing cells were diluted with sterile medium to theoretical densities of a single cell ml^{-1} which was then incubated in Durham tubes at 32 °C in 100 μmol photon m^{-2} s^{-1}. 20 replicates for each of ten strains were tested, but in no case did any of the cultures grow from single cells.

2.823 Streaking

Logarithmically-growing cultures were streaked on nutrient agar plates and incubated at 32 °C in 100 μmol photon m^{-2} s^{-1} for 3 - 4 weeks. Colonies which grew on the agar were removed using sterile micropipettes and resuspended in liquid medium. This method was unsuccessful with all ten strains which were tested.

2.824 Flow cytometry

The author obtained the idea that he might be able to use the cell sorting facility on some flow cytometers to separate fluorescing (autotrophic cells) from non-fluorescing cells (bacterial contaminants). Contaminated cultures (all derived from the enrichment culture and subsequently plated out on agar following the aerosol method - see above) which were in early exponential phase of the growth curve, were diluted with sterile medium to densities of

10^4 cells ml^{-1}. Fluorescing particles (autotrophic picoplankton) were then separated from non-fluorescing particles (bacteria) using a Beckton-Dickinson FACS 420 laser flow cytometer. Samples were excited with 500 mW of 514 nm wavelength light from a water-cooled Argon laser and fluorescence emission was measured at 660-700nm (red). Two parameters were selected for measuring cells passing through the laser beam: fluorescence and forward angle light scatter (which gives an estimate of cell size). Cells with a strong fluorescence and relatively large cell size were electronically "gated" and cells in this "window" were sorted. Approximately 5000 sorted cells were collected in Durham tubes containing sterile media; these were subsequently incubated at 32 °C in 100 μmol photon m^{-2} s^{-1}. Ten replicates per strain were treated in this way. Cultures which grew were plated on bacterial test plates and in all replicates there was evidence of some contamination though at a much reduced level compared with pre-sorted cultures. The sorting process was repeated with the "improved" cultures with one modification: each culture was sorted three times ie sorted cells were sorted again and then again. In four strains this produced axenic cultures (D774, D798, D799 and D838) and in two others (D807 and D772) a subsequent repitition of the aerosol method rendered strains axenic.

CHAPTER 3

METHODOLOGY INVESTIGATIONS

3.1 INTRODUCTION

No definitive autotrophic picoplankton methodology has been described in the literature. Most researchers use variations of a common epifluorescence microscopical procedure (Section 1.73) but since filter sets vary according to the type of microscope used, visible fluorescence properties of cells differ from laboratory to laboratory. The aims of the studies in this chapter were to devize a standard methodology which would be used throughout the research project.

3.2 PRESERVING AUTOTROPHIC PICOPLANKTON CELLS

3.21 Introduction

Samples would often be counted up to 12 h after sampling so it was important to establish the efficacy of preserving cells for subsequent enumeration. Few preservation studies had been published prior to 1986 and none included long term preservation. Initial observations of field samples revealed two types of autofluorescing cyanobacteria: red-fluorescing and orange-fluorescing. Both types were used in the preservation study. Samples were not frozen as the study aimed to provide a method which could be used by expeditions without access to expensive equipment.

Fig. 3.1 Cell counts of autotrophic picoplankton preserved with 2% buffered formalin or glutaraldehyde in the dark at 4 °C for 12 months.

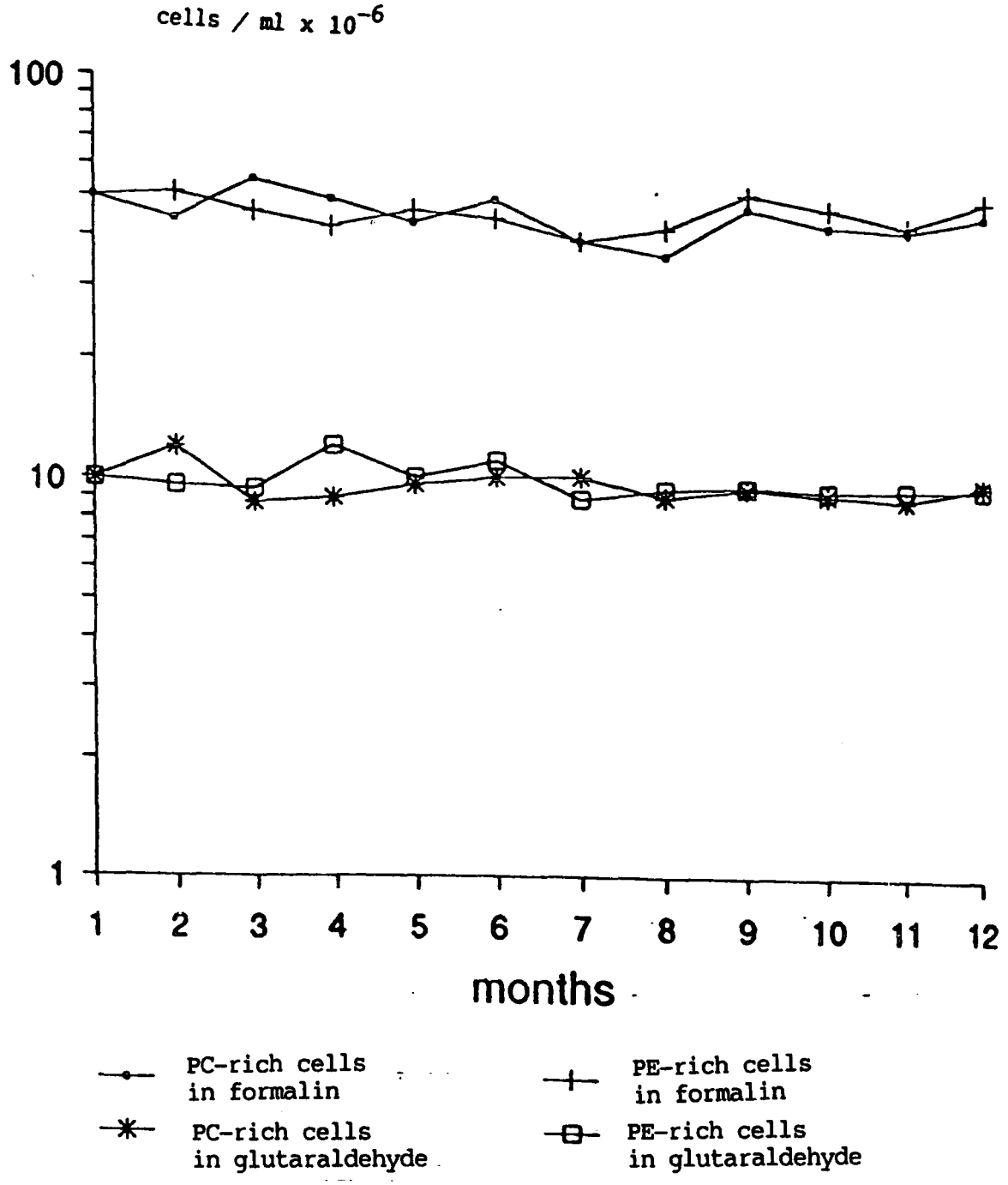


Table 3.1 Fluorescence intensity of cultured autotrophic picoplankton cells under different excitation filter sets in logarithmic growth phase and nitrogen limitation. +++ = strong fluorescence; ++ = visible; + = weak fluorescence; - = no fluorescence

Strain	Physiological status of cell	Culture to naked eye	Fluophot filter set Green	Blue
D 797	log	blue-green	+++	-
	N-deficient	green	++	-
D 548	log	green	+	+++
	N-deficient	green	+	+++
D 562	log	blue	+++	-
	N-deficient	blue-green	+++	-
D810	log	red	+++	++
	N-deficient	red	+++	++

3.22 Method

A phycocyanin-rich culture (D767) and phycoerythrin-rich cells from a sample from Ennerdale Water were preserved in 2% (final concentration) buffered (25 mM HEPES pH 7.0) glutaraldehyde or formalin. Cells were stored in the dark at 4 °C. Samples from the preserved material were withdrawn at monthly intervals and counted using the epifluorescence method described in Section 2.22.

3.23 Results

The results are presented in Fig. 2.1. Both types of cell (phycocyanin-rich and phycoerythrin-rich retained their fluorescence properties for long periods of time (up to 12 months) when stored at 4 °C in the dark and there was no obvious drop in the epifluorecence count.

3.3 FLUORESCENCE PROPERTIES OF CELLS

3.31 Introduction

Different genera of algae and cyanobacteria contain different pigment suites. Observations of cells in my own samples and reports in the literature (Section 1.5) suggested that both prokaryotic and eukaryotic autotrophic picoplankton may be present in freshwater samples. Red fluorescence is predominant in eukaryotic algae (chlorophyll *a* autofluorescence) and cyanobacteria when the main light harvesting pigment is phycocyanin. Distinguishing these in field populations could have been potentially difficult.

A study was carried out to devize a method to distinguish eukaryotic and prokaryotic autotrophic picoplankton using different filter sets on the

Fluophot microscope.

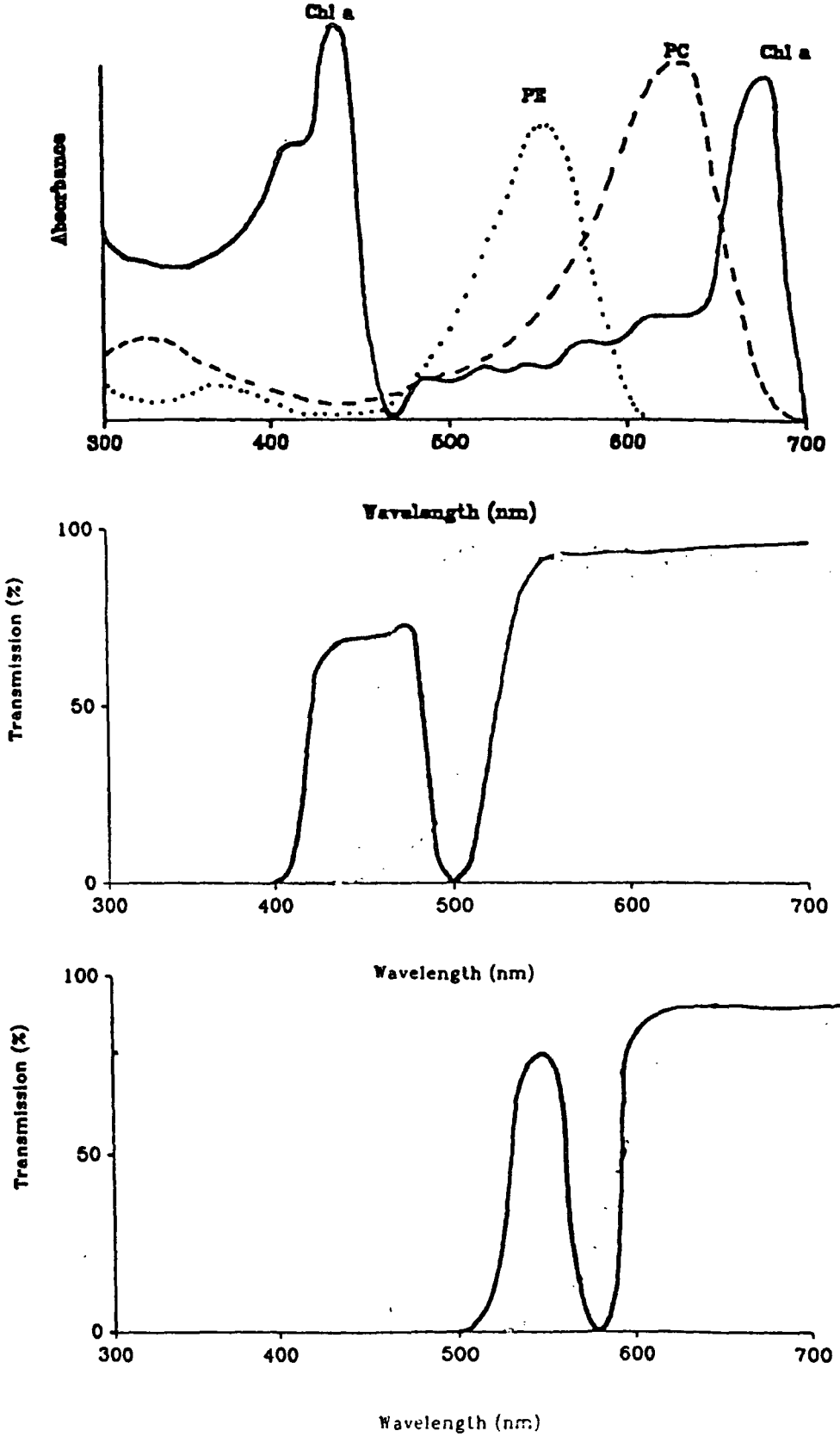
3.32 Method

A phycocyanin-rich culture (D562), a phycoerythrin-rich culture (D810), a Chlorella culture (D546) and a culture isolated from a deepwater rice environment (D797) were examined under epifluorescence microscopy at two physiological stages: logarithmic growth and N-deficiency. This was because Wyman et al. (1985) hypothesised that the phycobiliproteins may act as a nitrogen store in cells. If the hypothesis is true then cells which are nitrogen limited would have little accessory pigment and this could affect the identification of a cell if based on the fluorescence properties of the phycobilin pigment. Slides of the material were viewed under epifluorescence microscopy and the fluorescence intensity of cells in several fields of view were subjectively recorded on a 1-3 (brightest fluorescence = 3) scale under the green and blue excitation filter sets of the Fluophot microscope.

3.33 Results

The results are presented in Table 3.1; they indicated that cyanobacteria and eukaryotes can be distinguished by using the green and blue filter sets on the Fluophot microscope. Eukaryotic cells (D546) fluoresced more intensely under the blue excitation filter sets and were only just visible when excited with green light. Phycocyanin-rich cells exhibited the same brick red fluorescence, but under different excitation conditions: they were barely visible under blue excitation but fluoresced strongly when excited with green light. Phycoerythrin-rich cells fluoresced bright orange under

Fig. 3.2 The absorption characteristics of photosynthetic pigments and the optical properties of the filter sets used for epifluorescence microscopy in the Fluophot microscope. Top: absorption spectra of pigments (Craig, 1984): chlorophyll a (chl a), phycocyanin (PC) and phycoerythrin (PE). Middle: transmission properties of the blue excitation filter set on a Nikon fluophot microscope. Bottom: transmission properties of the green excitation filter set



green excitation and a dull yellow under blue excitation. Thus prokaryotic autotrophic picoplankton could be distinguished best under green excitation and eukaryotic cells under blue excitation. Fig. 3.2 helps to explain the results in Table 3.1; the graphs show that the excitation filter sets overlap with specific regions of the pigment absorption spectra: the blue filter set excites the shorter wavelength peak of the chlorophyll a absorption spectrum and the green filter set overlaps with the phycocyanin and phycoerythrin absorption peaks but not either of the chlorophyll a peaks.

3.4 FLUORESCENCE FADING

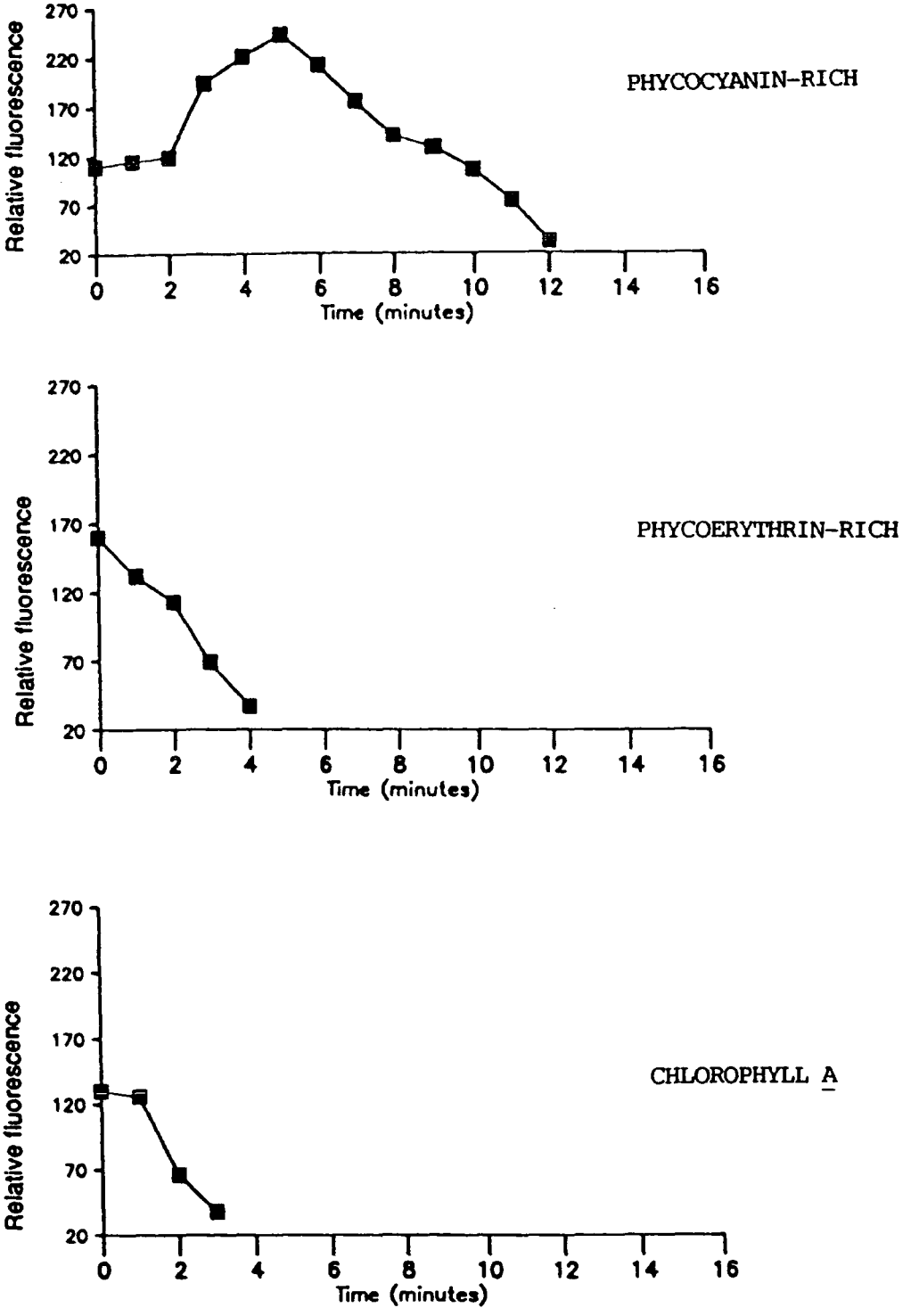
3.41 Introduction

Observations of slides of cultured Chlorella cells and phycocyanin-dominant cyanobacteria revealed that the fluorescence from eukaryotic cells faded faster than fluorescence from the cyanobacteria. An investigation was carried out to quantify this effect with the aim of using any differences as a supplementary means of distinguishing prokaryotic from eukaryotic autotrophic picoplankton.

3.42 Method

Preserved cells of D768 (phycocyanin-rich cyanobacterium), D546 (chlorophyll a-rich) and phycoerythrin-rich cells from Ennerdale Water were examined under epifluorescence microscopy with a microscope photometer attachment. Time courses of fluorescence intensity were measured for each sample. Six cells from each group were recorded.

Fig 3.3 Time course of fluorescence intensities in strains of autotrophic picoplankton. The top graph shows the intensities of phycocyanin-rich cells (strain D768), the middle graph shows intensities of phycoerythrin-rich cells (from Ennerdale Water) and the bottom graph shows intensities of Chlorella cells (D546)



3.43 Results

The results are presented in Fig. 3.3. Chlorophyll a dominant cells show rapid fluorescence fading whilst phycocyanin-rich cells show a gradual fluorescence intensity increase before fading. Phycoerythrin-rich cells behave in the same way as chlorophyll-rich cells with respect to fluorescence fading.

3.5 DISCUSSION

Results in this chapter showed the efficacy of preserving cells over long periods. This was useful for counting autotrophic picoplankton several days or weeks after sample collection. Comparisons of the fluorescence characteristics of cells under different excitation filter sets and fluorescence-fading time course studies indicated that a distinction could be made between prokaryotic and eukaryotic picoplankton, although a degree of subjectivity was required.

The results formed the basis of the methodology which was used for the rest of the studies. Samples were invariably preserved with buffered 2% formalin and the distinction made between prokaryotic and eukaryotic autotrophic picoplankton (see Chapter 5) was achieved using the different filter combinations and observing fluorescence fading (though a photometer was not used routinely).

CHAPTER 4

AUTOTROPHIC PICOPLANKTON DENSITIES FROM FIVE CONTINENTS

4.1 INTRODUCTION

Having established a methodology to count and preserve autotrophic picoplankton, surveys were carried out to determine how widepsread autotrophic picoplankton are in freshwaters. Most freshwater autotrophic picoplankton studies reported in the literature concern detailed studies of one particular water body. Reviewers have pooled reports and have started to get an idea of global distribution (Section 1.61) but there have been no broad surveys of freshwater autotrophic picoplankton carried out by indivdual investigators using one consistent methodology. Results from the preservation studies in the previous chapter (Section 3.2) demonstrated the efficacy of preserving samples for subsequent enumeration in the laboratory weeks or months later. This enabled samples to be collected from remote areas for an investigation of autotrophic picoplankton distribution over a large geographic area. Studies in this chapter were carried out to assess autotrophic picoplankton densities from freshwaters around the world and from freshwaters in northern England and Scotland.

4.2 AUTOTROPHIC PICOPLANKTON DENSITIES FROM FIVE CONTINENTS

Freshwater samples from around the world were collected and returned to the U.K. for subsequent enumeration. Most samples were collected by expeditions or friends travelling abroad. All samples were stored in the

Table 4.1 Autotrophic picoplankton densities in standing waters from around the world

Country	Water body	Location	Sampling date	Cells ml^{-1}
Austria	Wörther See	46°20'N 14°E	01/07/89	2.86×10^3
Australia	Lake Samsonvale	27°30'S 150°E	29/08/88	3.01×10^2
Bolivia	Lake Titicaca	15°30'S 68°50'W	17/01/88	4.56×10^4
Canada	Lake Simcoe	44°N 79°W	14/06/88	3.00×10^3
	Lake St Clair	42°30'N 82°40'W	22/06/88	4.86×10^4
Crete	Lake Kournes	35°5'N 25°30'W	15/04/88	6.80×10^4
India	Surha Tal	25°50'N 84°10'E	04/10/88	3.86×10^4
	Dal Lake		18/10/88	3.64×10^4
	Manasbal Lake	lie within:	18/10/88	1.03×10^5
	Wular Lake	32°17' - 37°51'N	19/10/88	6.53×10^4
	Lake Gangebal	and	26/10/88	4.34×10^5
	Lake Anchar	72°40' - 80°30'E	30/10/88	6.80×10^2
	Nilnag Lake		03/11/88	3.32×10^2
Ireland	Lough Graney	53°N 9°25'W	17/03/88	6.26×10^2
Italy	Lago di Bracciano	45°7'N 11°50'E	19/09/88	7.63×10^4
	Lago di Mortignano		15/09/88	5.41×10^4

Table 4.1 (cont'd)

Country	Water body	Location	Sampling date	Cells ml^{-1}
Jordan	Azraq Oasis	31°50'N 36°40'	17/03/88	2.05×10^3
Kenya	Lake Victoria	0°35'S 34°30'E	16/08/87	3.70×10^4
Madagascar	Lake Alaotra	17°S 49°E	13/08/88	2.06×10^2
Mallorca	Embalse de Gorge Blau	39°N 3°E	21/04/88	5.64×10^3
Nepal	Narayan Hiti Pokhari Nag Pokhari Naxal	27°N 85°E	10/06/88 12/06/88	2.10×10^2 3.31×10^2
New Zealand	Lake Ohau	44°10'S 170°E	29/08/88	2.00×10^3
Norway	Lake Leirvatnet	59°N 7°E	31/08/88	1.34×10^4
Saudi Arabia	Pool	no data	21/03/88	1.02×10^2
Sweden	Lake Mälaren	59°30'N 17°0'E	29/07/88	3.08×10^3
Switzerland	Lake Geneva	34°N 80°E	23/06/89	8.63×10^3
United Kingdom	Ennerdale	54°40'N 3°25'W	15/09/88	1.20×10^6
U.S.A.	Mountain Lake	37°8'N 80°30'W	27/10/87	7.20×10^3

dark until enumeration, most were refrigerated for some or all of their time in storage. All samples were enumerated within one month of collection. The results are presented in Table 4.1.

4.21 RESULTS

Every sample collected across five continents contained autotrophic picoplankton. The minimum cell density recorded was 1.02×10^2 cells ml^{-1} from a pool in Saudi Arabia and the maximum density recorded was 1.20×10^6 cells ml^{-1} recorded in Ennerdale Water in the UK. No environmental data were collected so no correlations with other variables could be determined. In addition, the samples were collected at different times of the year, so it was impossible to glean any information concerning seasonal trends. However, the study showed that freshwater autotrophic picoplankton are apparently ubiquitous.

4.3 AUTOTROPHIC PICOPLANKTON DENSITIES IN U.K. WATERS

The study in Section 4.2 established the widespread distribution of picoplankton from around the world. However, to try and establish whether autotrophic picoplankton differ from one type of water body to another and to see whether or not populations change seasonally it is necessary to compare populations from different water bodies at the same time of the year and study populations over at least a year. In order to identify sites for a closer study of seasonal trends in autotrophic picoplankton a preliminary survey of thirty lakes in northern England and Scotland was carried out.

4.31 Lentic environments

A survey of thirty lakes in north England and Scotland was carried out. Sites were selected which were within reasonable travelling distance of Durham or collected whilst involved with undergraduate field trips. Samples were collected from a single sampling site and preserved in 2% buffered formalin. Results are presented in Table 4.2.

Table 4.2 Densities of autotrophic picoplankton in 30 freshwater bodies in the UK.

Water body	Location West	Location North	Sampling date	Picoplankton density (cells ml^{-1})
Balmaha Pond	4°31′	56° 6′	22/04/87	2.45×10^2
Bassenthwaite Lake	3°10′	54°40′	16/05/87	3.45×10^3
Buttermere	3°15′	54°35′	16/05/87	5.10×10^4
Brasside Pond	1°35′	54°40′	18/03/87	2.65×10^2
Cassop Pond	1°38′	54°43′	18/03/87	3.37×10^2
Coniston Water	3° 5′	54°20′	17/05/87	8.76×10^4
Croft Kettle	1°35′	54°20′	18/03/87	2.57×10^2
Crummock Water	3°20′	54°35′	16/05/87	2.34×10^5
Derwent Reservoir	1°50′	54°50′	16/05/87	1.34×10^2
Derwentwater	3° 8′	54°35′	02/04/87	3.89×10^3
Dubh Loch	4°35′	56° 8′	19/04/87	1.68×10^3
Ennerdale Water	3°26′	54°32′	17/05/87	4.26×10^5
Esthwaite Water	2°58′	54°20′	17/05/87	1.02×10^2
Hatfield Pond*	1°38′	54°40′	18/03/87	3.45×10^3
Hell Kettle	1°35′	54°25′	18/03/87	5.43×10^4
Lake of Menteith	4°15′	56°10′	20/04/87	3.68×10^3

Loch Achray	4°24′	56°14′	20/04/87	5.42×10^2
Loch Ard	4°28′	56°12′	20/04/87	3.89×10^3
Loch Arklet	4°38′	56°15′	20/04/87	3.89×10^2
Loch Hope	4°35′	58°25′	03/05/88	7.34×10^3
Loch Katrine	4°38′	56°18′	06/05/88	3.56×10^3
Loch Lomond	4°35′	56°10′	22/04/87	4.55×10^3
Loch Loyal	4°25′	58°25′	03/05/88	1.02×10^4
Loch Lubnaig	4°15′	56°15′	20/04/87	2.63×10^2
Loch Naver	4°30′	58°20′	03/05/88	3.32×10^3
Loch Shin	4°45′	58° 5′	02/05/88	6.45×10^4
Loch Venachar	4°20′	56°10′	20/04/87	3.75×10^2
Malham Tarn	2°10′	54° 5′	13/04/87	4.67×10^3
Ullswater	2°55′	54°35′	16/05/87	3.45×10^3
Wastwater	3°18′	54°26′	17/05/87	1.59×10^5

* Pond was drained in November 1988.

Samples were collected between March and May (although not all in the same year) in order to minimise seasonal differences between samples.

Densities ranged from a minimum of 1.02×10^2 cells ml^{-1} in relatively eutrophic Esthwaite Water to a maximum of 2.34×10^5 cells ml^{-1} in relatively oligotrophic Crummock Water.

4.32 Lotic environments

The studies reported in Sections 4.1 and 4.2 revealed that autotrophic picoplankton were apparently ubiquitous in freshwater standing waters. However, all the samples taken in this study were from standing freshwaters

and since there are no reports in the literature of autotrophic picoplankton densities in flowing waters, samples from a number of rivers were collected. Four rivers were chosen and these could be split subjectively into two categories at the sampling points: fast flowing mountain streams (R. Ehen and R. Liza) and slow flowing rivers (R. Tees and R. Tyne). No attempt was made to cover a spectrum of river flows and conditions, rather a comparison was made between two different lotic habitats. Samples were collected in July 1989 (R. Ehen, R. Tees, R. Liza) and in August 1989 (N. Tyne) from just below the water surface, preserved in 2% buffered formalin and counted in the laboratory. The results are presented in Tables 4.3 - 4.6.

Table 4.3 Autotrophic picoplankton densities in R. Ehen at different distances from Ennerdale Water. The proportion of the cell which fluoresced was determined subjectively in 20 randomly chosen cells in each sample, and the mean area of cellular fluorescence was put into one of three classes: +++ = 100% cell fluorescence; ++ = <50% cell fluorescence; + = <20% cell fluorescence.

Site	Grid reference	Distance from lake (km)	Density (cells ml^{-1})	% cell area fluorescing	%PC-rich cells
1	NY 070158	4	3.25×10^4	+++	0
2	NY 029144	9	7.89×10^4	++	3
3	NY 012104	13	4.67×10^4	+	2
4	NY 006060	18	6.47×10^3	+	5
5	NY 024027	22	1.02×10^1	+	1

Many of the cells lacked the uniform fluorescence characteristic of all the samples examined from standing water bodies. The amount of cellular fluorescence decreased with increasing distance from Ennerdale Water and the overall density of cells decreased too. Most of the cells in all the

samples were phycoerythrin-rich.

Table 4.4 Autotrophic picoplankton densities in R. Liza.

Site	Grid reference	Density (cells ml^{-1})
1	NY 192123	<10
2	NY 180130	<10
3	NY 155139	<10
4	NY 131143	<10

No autotrophic picoplankton cells were detectable in any of the samples.

Table 4.5 Autotrophic picoplankton densities in R. Tees. The proportion of fluorescence was subjectively assessed in 20 randomly chosen cells in samples from each site and the mean area of fluoresence was put into 1 of 3 classes: +++ = 100% cell fluorescence; ++ = <50% cell fluorescence; + = <20% cell fluorescence.

Site	Grid reference	Density (cells ml^{-1})	Appearance of cells	% PC-rich cells
1	NZ 474195	2.36×10^2	+++	90
2	NZ 465193	1.02×10^2	+++	96
3	NZ 460191	4.56×10^2	+++	81
4	NZ 455191	7.98×10^2	+++	93
5	NZ 450193	5.42×10^2	+++	91

Autotrophic picoplankton densities increased on passing downstream and unlike cells in R. Ehen, all cells showed uniform fluorescence. The majority of cells in each sample were phycocyanin-rich.

Table 4.6 Autotrophic picoplankton densities in R. North Tyne at different distances from Kielder Water.

Site	Grid reference	Distance from reservoir (km)	Density (cells ml^{-1}) 25/07/89	25/08/89	%PC-rich cells 25/07	25/08
1	NY 713874	1.0	0.49×10^2	0.10×10^2	8	5
2	NY 723872	2.5	2.12×10^2	0.74×10^2	12	10
3	NY 744858	5.5	6.45×10^2	1.28×10^2	16	12
4	NY 809846	14.5	6.11×10^2	4.15×10^2	38	26
5	NY 834833	18.0	15.3×10^2	5.57×10^2	49	52
6	NU 919706	38.0	28.5×10^2	9.05×10^2	63	59

On both sampling dates autotrophic picoplankton densities increased with increasing distance from Kielder Water; in July 1989 the difference in autotrophic picoplankton density between the first and the sixth site was 2,800 cells ml^{-1} and the following month was 900 cells ml^{-1}. On both sampling dates the proportion of phycocyanin-rich picoplankton increased on passing downstream by about an order of magnitiude.

4.4 COMMENT

Autotrophic picoplankton were detected in all the standing freshwaters sampled. The apparent ubiquity of these organisms mirrors the situation in the marine environment where every sample enumerated for autotrophic picoplankton contained these cells (Section 1.61). The results of the survey of lakes in the U.K. seemed to indicate a relationship between lake trophic status and autotrophic picoplankton density.

The only flowing water in which autotrophic picoplankton was not detected was R. Liza; this was the only river which does not have a standing water in

its catchment. The other 3 rivers have a lake or reservoir upstream of the sampling points and all samples contained autotrophic picoplankton. The autotrophic picoplankton population only increased in size noticeably between the upstream sampling point and the furthest downstream sampling point in R. North Tyne. In R. Liza both the population size and the mean area of cellular fluorescence decreased between the upstream and downstream sampling points. Cells in the R. Ehen samples looked very different from any of the other samples examined throughout the whole study. Since cells in Ennerdale Water (the presumed source of inoculum of autotrophic picoplankton in R. Ehen) always showed 100% cell fluorescence, it is probable that cells in the river were damaged in some way; this hypothesis is supported by the fact that the population decreased with increasing residence time in the river. The nature of the reaches of the 3 rivers which contained autotrophic picoplankton are different. R. Ehen is a fast-flowing, turbulent stream, which may represent a poor environment for autotrophic picoplankton. In contrast, R. North Tyne and R. Tees are relatively slow flowing and perhaps provide an environment in which autotrophic picoplankton can survive and grow.

CHAPTER 5

AUTOTROPHIC PICOPLANKTON VARIATION IN TIME AND SPACE

5.1 INTRODUCTION

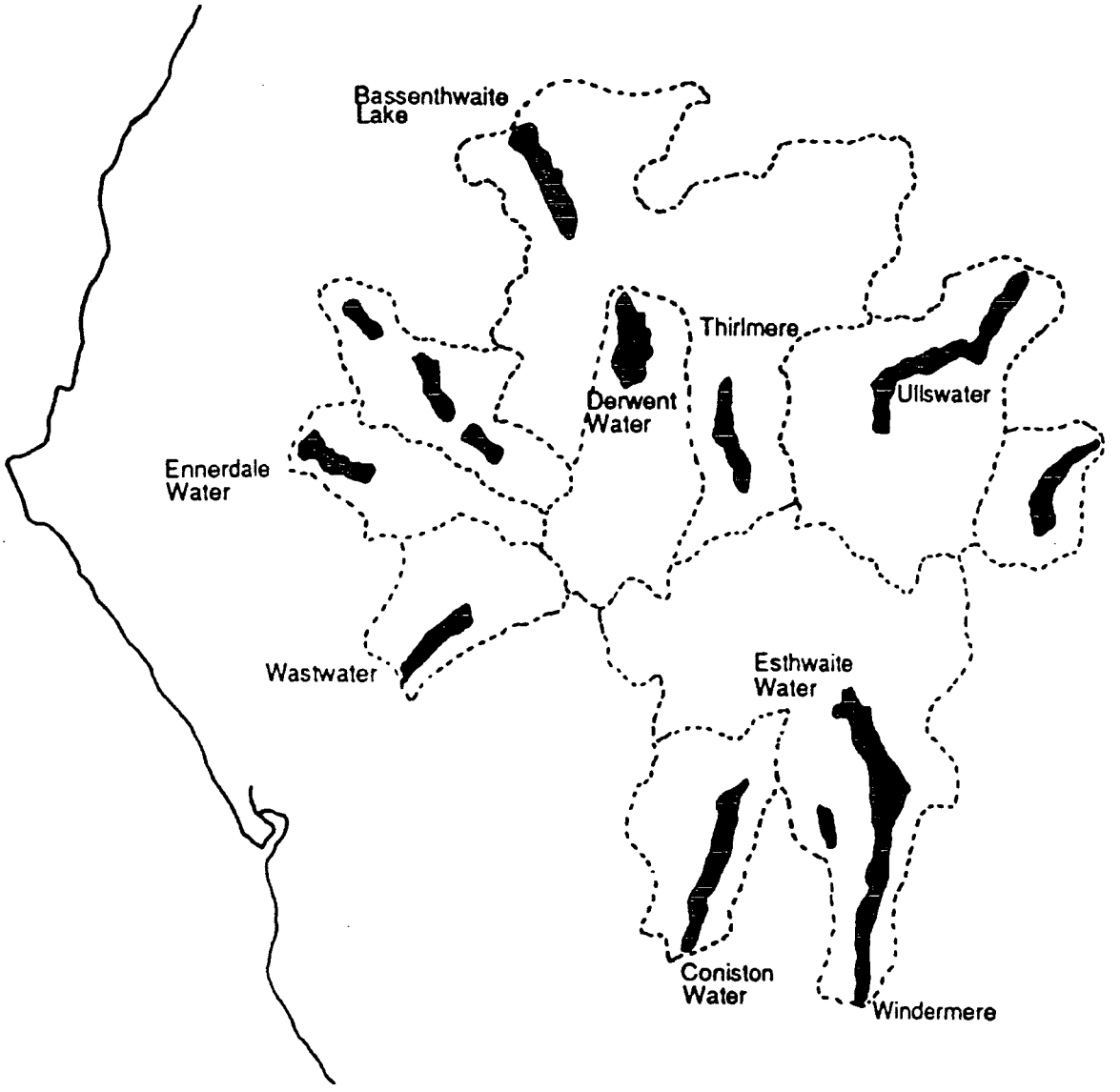
Studies in the previous chapter indicated that autotrophic picoplankton are widespread in freshwater environments. However, since sampling dates in Table 4.1 spanned a fifteen month period, direct comparison between water bodies is impossible. The literature review (Section 1.62) showed that seasonal variations of autotrophic picoplankton density have been recorded in a number of standing freshwaters. The survey of thirty lakes in the U.K. (Section 4.31) indicated that autotrophic picoplankton densities and lake trophic status may be related so a study was planned to examine temporal variations in ten lakes with different trophic status. Studies were also conducted to ascertain the effect of microhabitat on autotrophic picoplankton density.

5.2 TEMPORAL AUTOTROPHIC PICOPLANKTON VARIATION

5.21 Study sites

The main criteria for selecting lakes for temporal study was geographic proximity to Durham and trophic status. The Cumbrian lakes provided a range of different lake types and since these are situated in a relatively small area several lakes could be sampled on a single day. In addition Malham Tarn and Cassop Pond were studied. The location of the Cumbrian lakes is given in Fig. 5.1.

Fig. 5.1 Eight Cumbrian lakes studied for fourteen months, together with Windermere but which was not sampled. Dotted lines represent lake catchments. (after Ramsbottom, 1976)



5.22 Method

The trophic status of lakes was ascertained by literature search (Godinho-Orlandi & Jones, 1981) and confirmed by analysis of a single total filtrable phosphorus sample from each site collected in March 1988. Duplicate samples of autotrophic picoplankton were taken each month from a single station at each lake. The locations of sites are given in Table 5.1. Samples were collected over a fourteen month period as in seven lakes autotrophic picoplankton populations had not returned to previous values by the start of the following year. The extra samples were collected to ascertain whether autotrophic picoplankton densities returned to 1988 values by March 1989.

Table 5.1 Location and key morphometric features of lakes sampled; total filtrable phosphate (TFP) measurements were made in March 1988. Morphometric data are from Ramsbottom (1976).

Water body	Location		Area (km^2)	Max. depth (m)	TFP ($\mu g\ l^{-1}$)
	West	North			
Bassenthwaite Lake	3°13′	54°39′	5.28	19	13
Cassop Pond	1°27′	54°44′	0.05	c.2	38
Coniston Water	3° 5′	54°20′	4.91	56.1	5
Derwentwater	3° 9′	54°35′	5.35	22	12
Ennerdale Water	3°16′	54°32′	2.99	42	2
Esthwaite Water	2°59′	54°21′	1.00	15.5	26
Malham Tarn	2° 9′	54° 5′	0.62	4.4	25
Thirlmere	3° 4′	54°32′	3.26	46	4
Ullswater	3°53′	54°35′	8.94	62.5	8
Wastwater	3°13′	54°28′	2.91	76	2

5.23 Results

The environmental variables for the 10 lakes are presented in Table 5.2. All lakes were saturated with respect to oxygen for most of the year. Some lakes were supersaturated in the summer months, the highest recorded value being 131% saturation in Esthwaite Water in August. The minimum dissolved

Table 5.2 Monthly environmental variables in 1988 at the ten field sites

Water body	Variable	Month (1988) Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Bassenthwaite Lake	Dissolved oxygen (% saturation)	106	104	100	111	117	120	118	124	110	108	100	104
	Conductivity ($\mu S\ cm^{-1}$)	82.1	70.8	64.0	69.3	71.2	70.3	69.3	79.4	74.2	75.2	76.0	88.9
	pH	7.20	7.28	7.38	7.52	8.56	8.62	8.24	8.12	7.40	7.21	7.03	7.20
Cassop Pond	Dissolved oxygen (% saturation)	102	104	105	101	107	108	106	101	104	106	102	103
	Conductivity ($\mu S\ cm^{-1}$)	550	523	520	530	565	535	539	542	540	521	534	531
	pH	7.97	8.33	7.92	7.71	8.10	7.91	7.82	7.71	7.62	7.71	7.79	7.85
Coniston Water	Dissolved oxygen (% saturation)	103	105	103	104	116	106	111	109	116	104	100	102
	Conductivity ($\mu S\ cm^{-1}$)	67.9	71.0	66.9	67.1	73.1	69.2	70.2	70.9	66.4	69.2	72.0	72.9
	pH	7.00	7.04	7.05	7.13	7.37	7.24	7.30	7.19	7.14	7.20	7.11	7.15
Derwentwater	Dissolved oxygen (% saturation)	101	102	98	105	112	109	121	114	120	110	99	102
	Conductivity ($\mu S\ cm^{-1}$)	65.8	56.9	52.3	55.3	57.1	58.2	54.2	62.3	60.6	62.1	66.9	78.1
	pH	7.06	7.06	7.08	7.24	7.44	7.41	7.31	7.00	6.65	7.01	7.04	7.12
Ennerdale Water	Dissolved oxygen (% saturation)	104	102	99	103	106	108	104	110	106	104	98	102
	Conductivity ($\mu S\ cm^{-1}$)	51.7	45.9	48.8	47.4	46.7	45.4	42.1	42.1	46.3	44.2	44.9	49.4
	pH	6.69	6.51	6.62	6.59	6.56	6.62	6.57	6.41	6.62	6.54	6.78	6.77
Esthwaite Water	Dissolved oxygen (% saturation)	104	98	104	106	126	112	124	131	119	103	104	100
	Conductivity ($\mu S\ cm^{-1}$)	102	103	95.7	96.2	96.9	99.1	100	102	107	101	106	111
	pH	6.80	6.90	7.29	7.31	8.90	8.24	8.34	8.90	8.41	7.23	7.11	7.04
Malham Tarn	Dissolved oxygen (% saturation)	108	106	105	104	108	105	106	109	109	104	105	99
	Conductivity ($\mu S\ cm^{-1}$)	325	299	263	284	275	148	210	221	143	231	276	331
	pH	8.02	8.45	8.34	8.39	8.58	8.35	9.29	8.89	8.85	8.42	8.29	8.32
Thirlmere	Dissolved oxygen (% saturation)	103	108	101	108	113	119	108	105	100	102	104	101
	Conductivity ($\mu S\ cm^{-1}$)	49.1	45.1	39.2	40.2	43.2	44.3	39.5	40.2	43.4	46.2	47.6	51.3
	pH	6.76	6.80	6.52	6.61	6.83	6.92	6.50	6.61	6.72	6.91	6.88	6.71
Ullswater	Dissolved oxygen (% saturation)	100	104	103	111	125	119	124	113	106	108	103	102
	Conductivity ($\mu S\ cm^{-1}$)	76.2	66.5	66.9	64.1	62.5	72.1	74.3	72.9	77.1	75.2	74.8	74.6
	pH	7.28	7.30	7.05	7.08	8.33	8.15	8.21	8.04	7.41	7.11	6.86	7.10
Wastwater	Dissolved oxygen (% saturation)	100	102	100	114	105	107	106	100	102	104	98	100
	Conductivity ($\mu S\ cm^{-1}$)	57.2	54.0	59.8	46.9	52.1	53.4	49.6	52.1	53.6	56.2	43.7	50.3
	pH	6.50	6.68	6.48	6.72	6.66	6.34	6.47	6.31	6.47	6.68	7.00	6.27

oxygen value was 98% saturation and was recorded in four lakes: Derwentwater in March, Ennerdale Water in November, Esthwaite Water in February and Wastwater in November. In general, for individual lakes, dissolved oxygen saturations increased in the spring and summer (April - August); the largest difference between maximum and minimum dissolved oxygen concentrations was 33% in Esthwaite Water between February and August and the minimum difference was 7%, in Cassop Pond.

pH varied in lakes although all were circum-neutral. Comparison of January values showed that the lake with the lowest pH was Wastwater and the lake with the highest pH, Malham Tarn. There was a trend of increased pH in the spring and summer months in Bassenthwaite Lake with values rising to a maximum of 8.62 in June. Similarly in Esthwaite Water, pH values were higher in spring and summer compared with winter values; pH values in this lake reached peaks in May and August with values of 8.90. The highest pH recorded in the research period was 9.29 at Malham Tarn in July; like Bassenthwaite Lake and Esthwaite Water, pH values were higher in Malham Tarn in the spring and summer than in winter.

Conductivity remained relatively constant within lakes with the exception of Malham Tarn where values dropped throughout the year, reaching a trough in June and then rising again to a winter maximum. Of all the lakes studied, Ennerdale water had the lowest conductivity water and Cassop Pond the highest.

Comparison of some of the environmental variables, particularly dissolved oxygen and pH must be made with care since readings were taken at different times of the day. Individual lakes were sampled at the same time of day, but some were in the early morning (Ennerdale Water) and others around

midday (Wastwater, Cassop Pond, Ullswater, Thirlmere, Malham Tarn) or late afternoon (Esthwaite Water, Coniston Water, Bassenthwaite Lake, Derwentwater). Within a diel cycle dissolved oxygen and pH may be expected to reach a maximum in late afternoon as a result of photosynthesis.

Temperature, chlorophyll *a* , autotrophic picoplankton density and biovolume data collected at the ten lakes in 1988-89 are presented in Fig. 5.2.

A summary of the key features of the data is presented in Table 5.3.

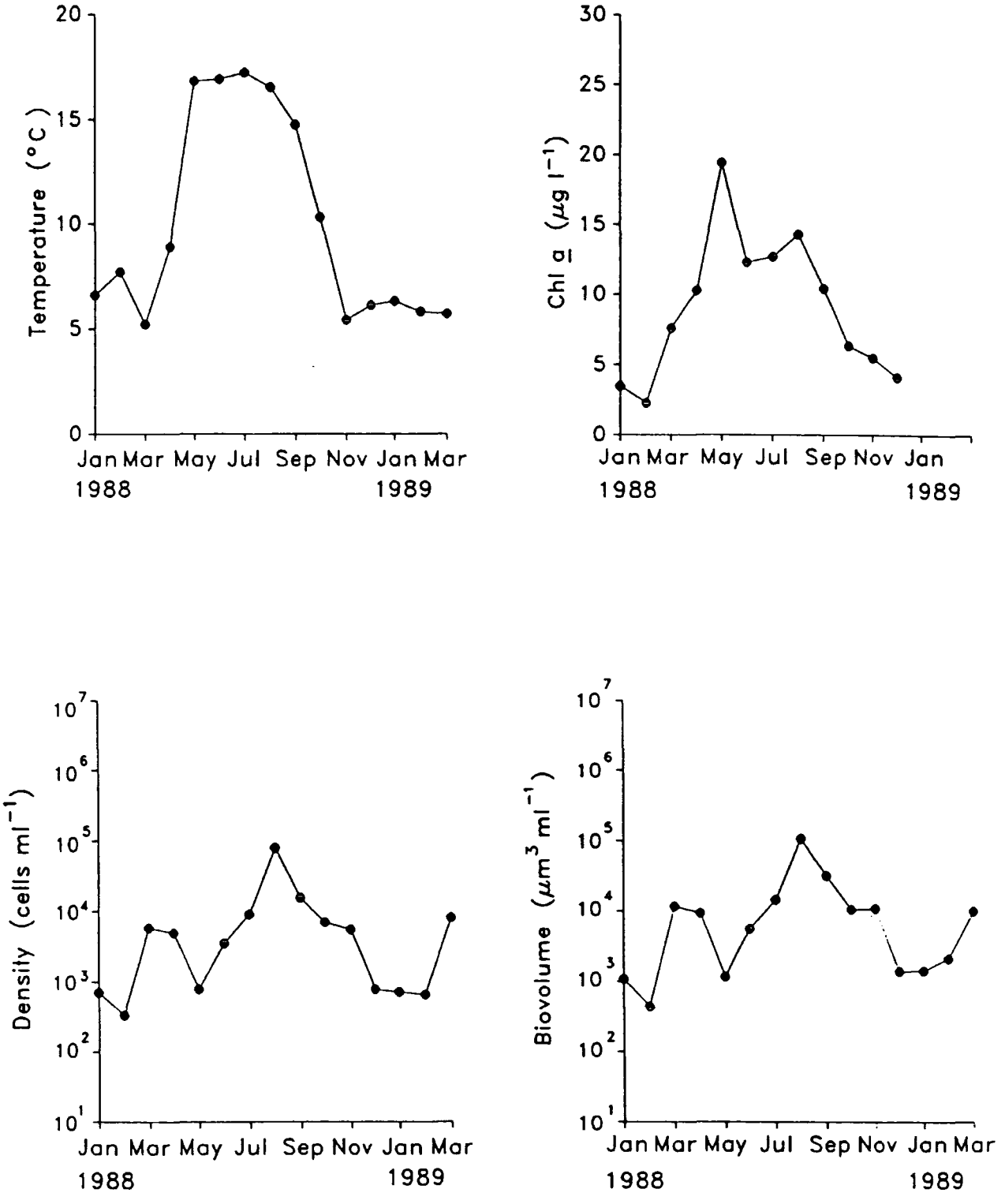
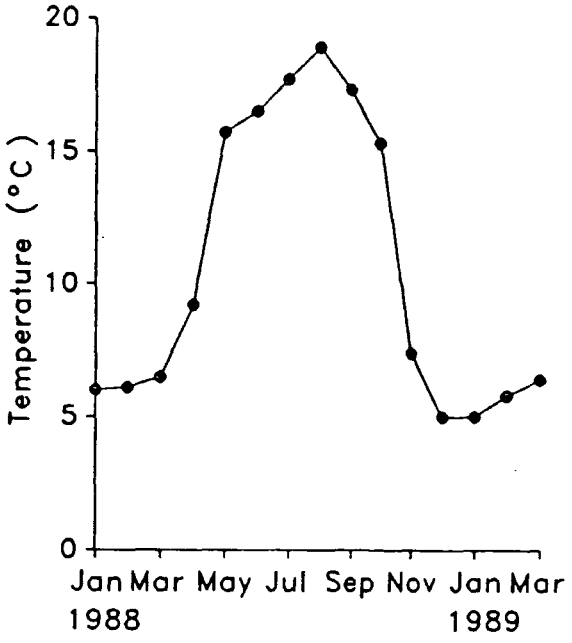
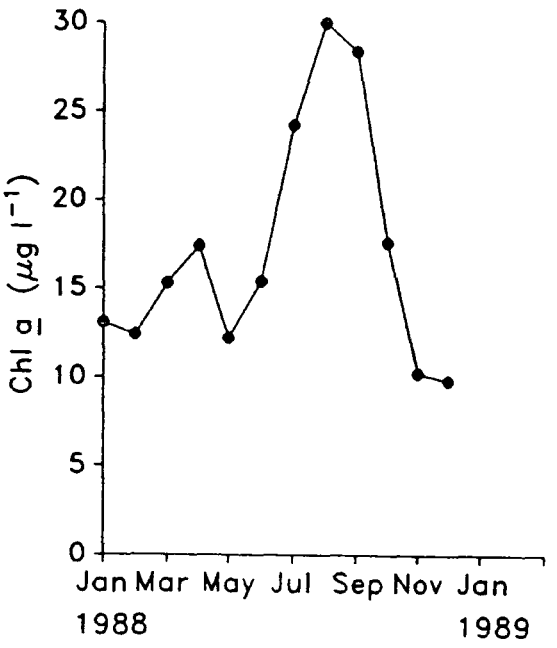
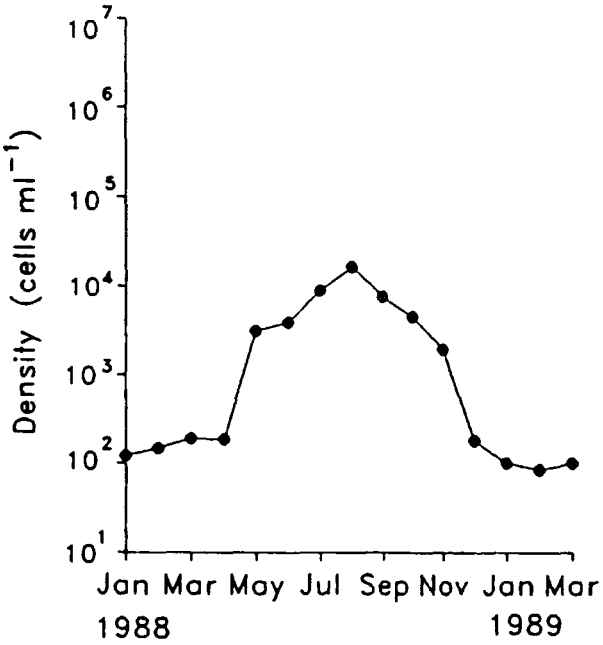


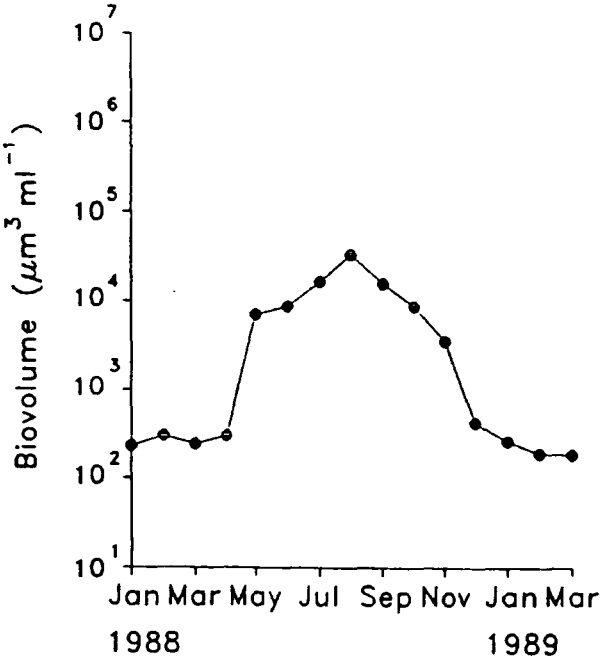
Fig 5.2 Temperature, chlorophyll a, autotrophic picoplankton biovolume and autotrophic picoplankton cell density in ten UK lakes

Cassop Pond

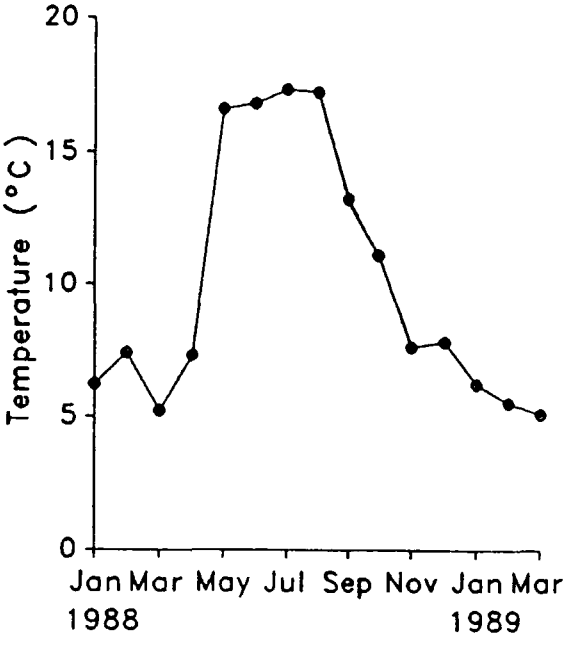

Temperature (°C)
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

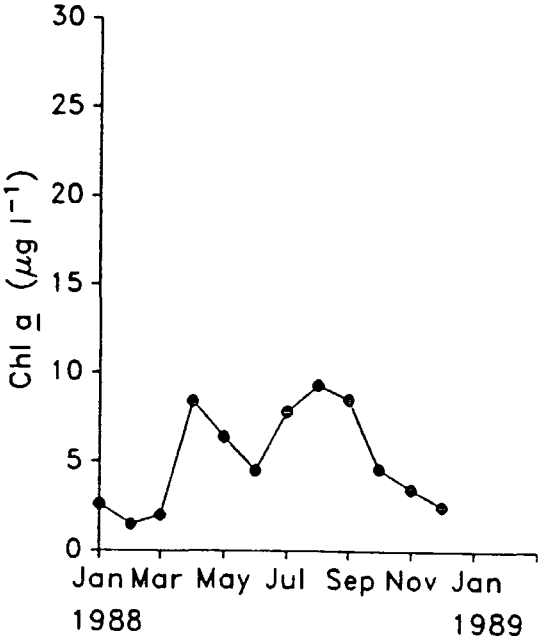

Chl a (μg l⁻¹)
30
25
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan
1988
1989

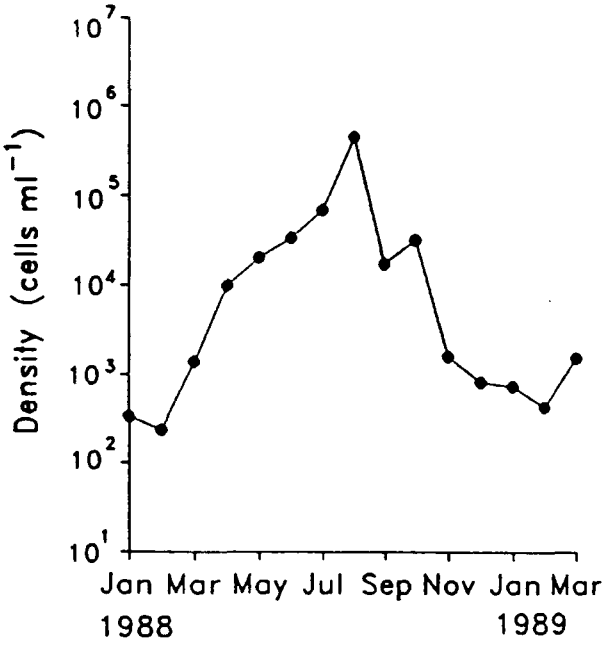

Density (cells ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

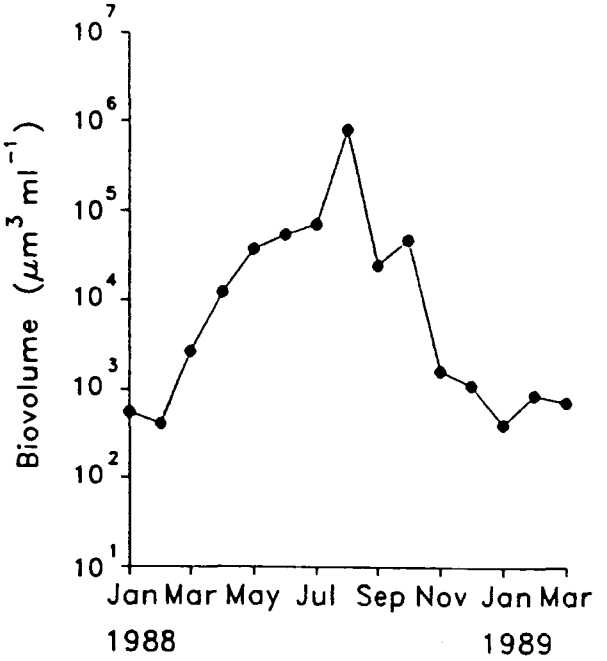

Biovolume (μm³ ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

Coniston Water

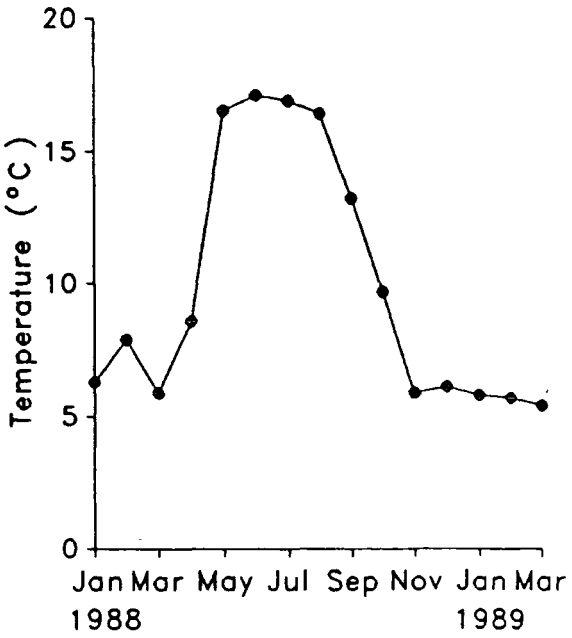


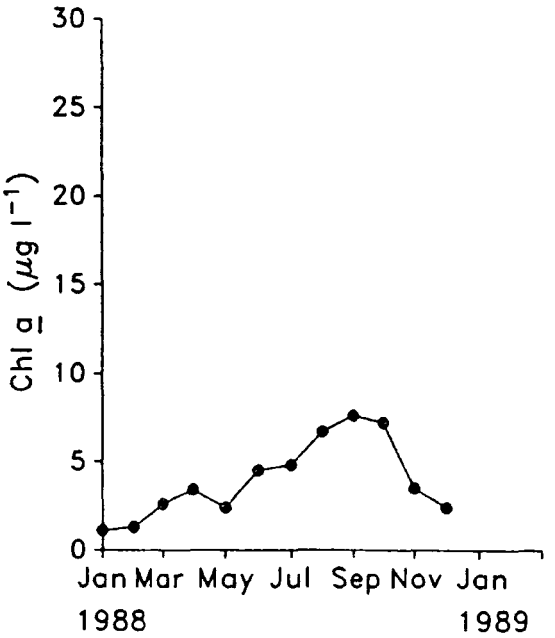


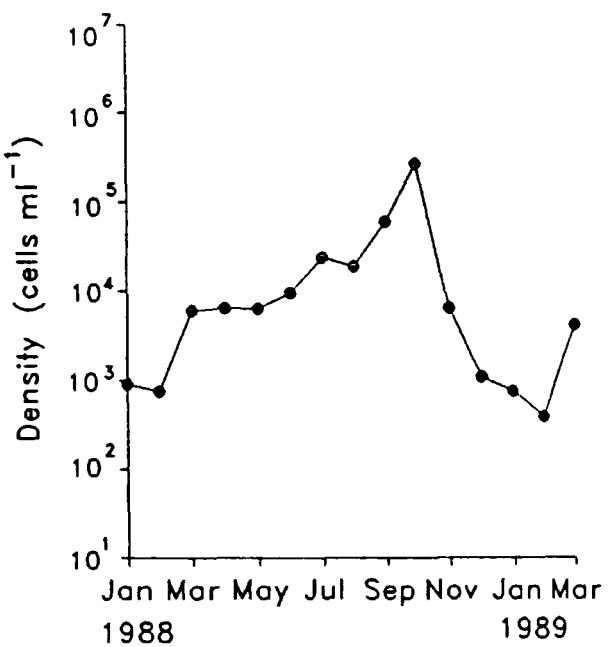


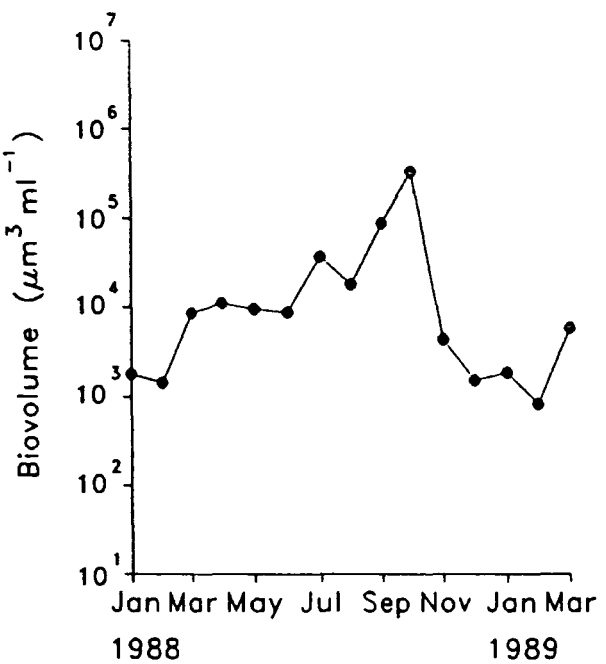


Derwentwater

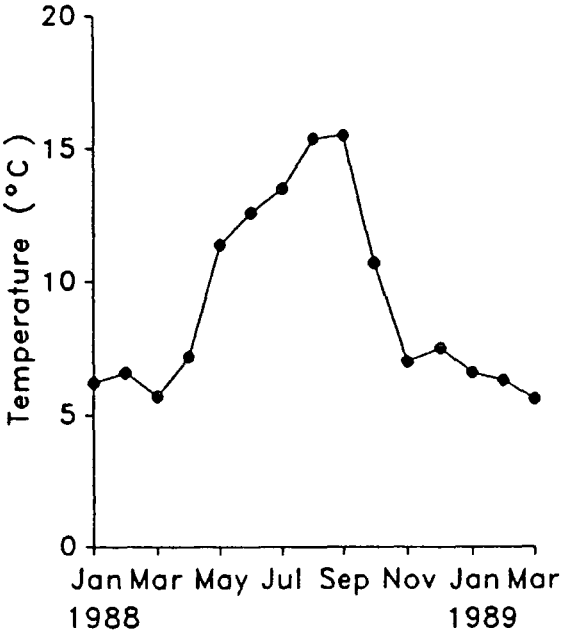


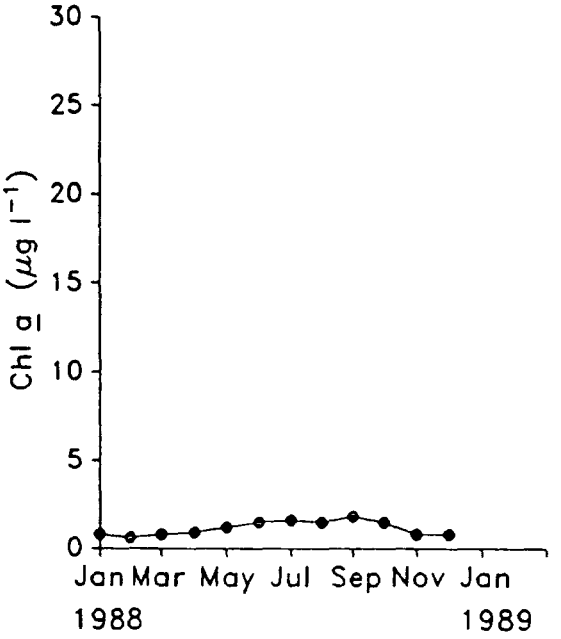


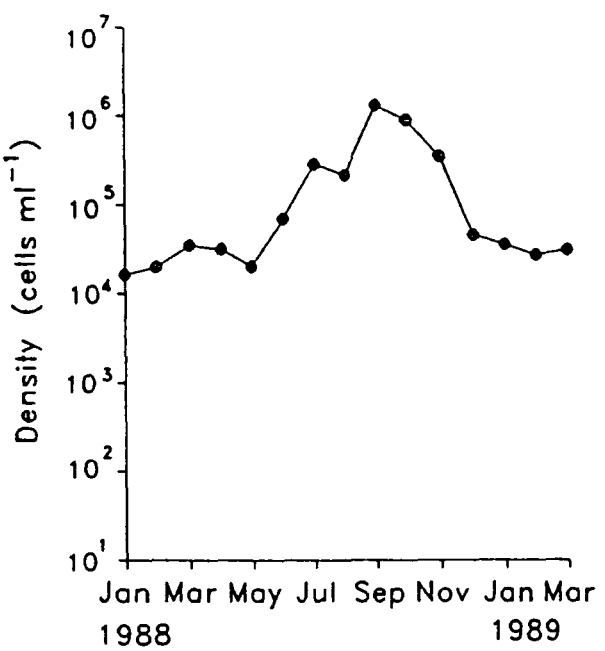


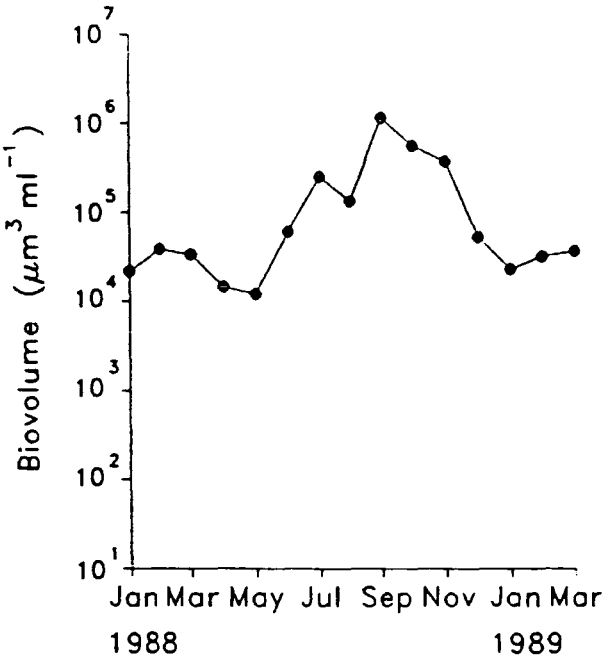


Ennerdale Water

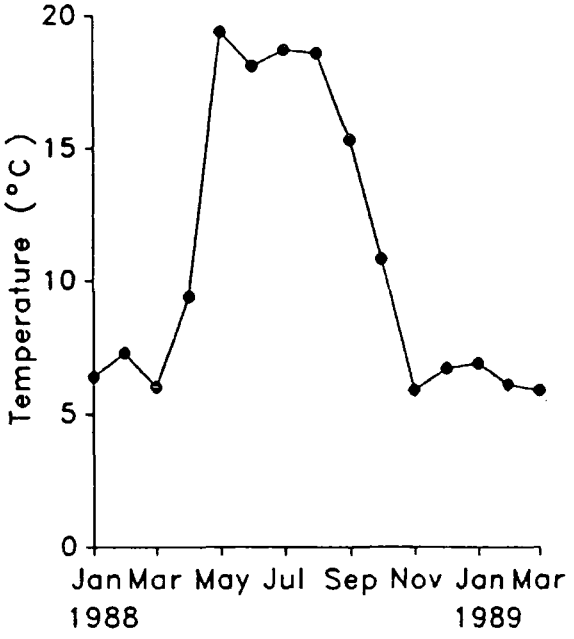


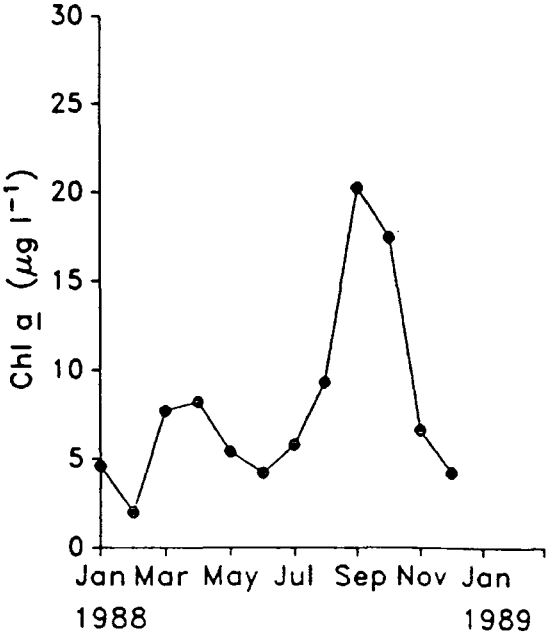


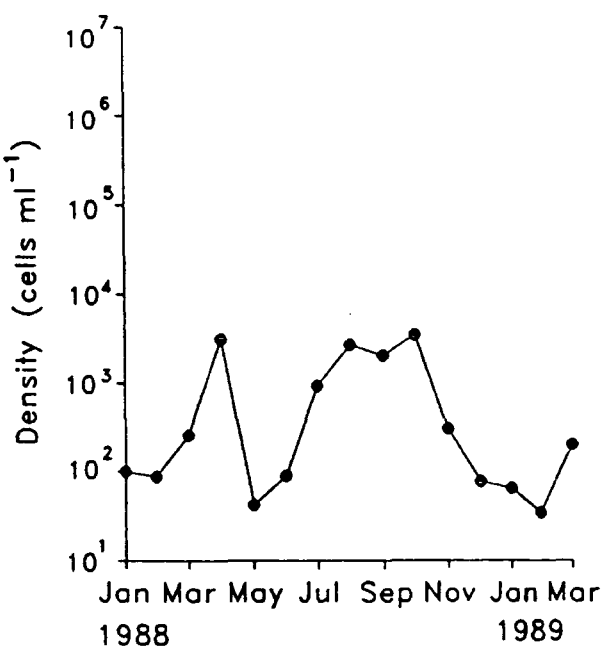


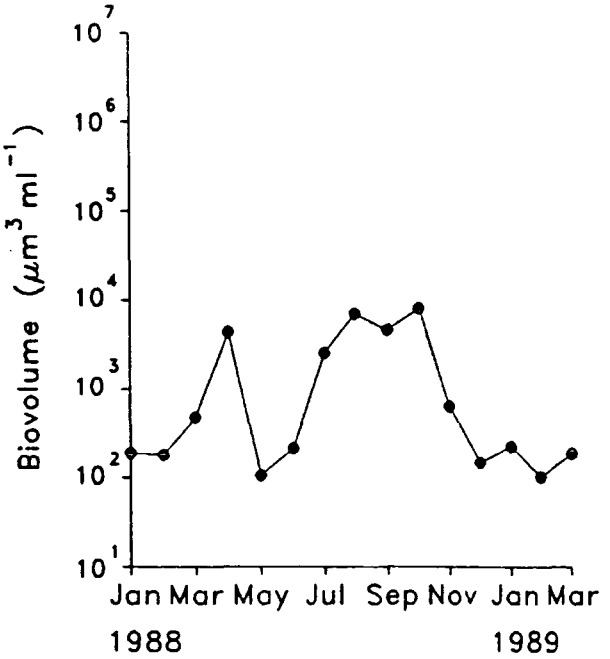


Esthwaite Water

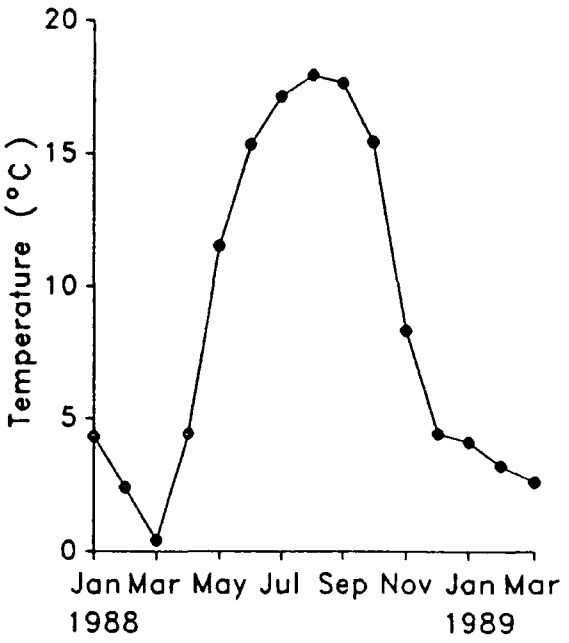


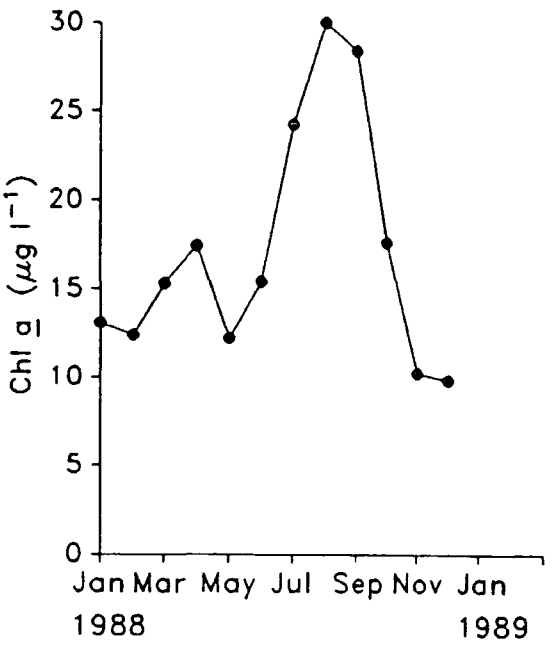


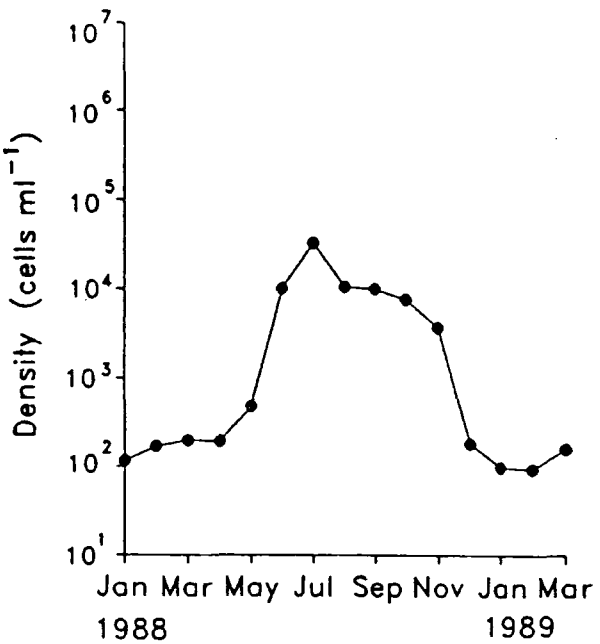


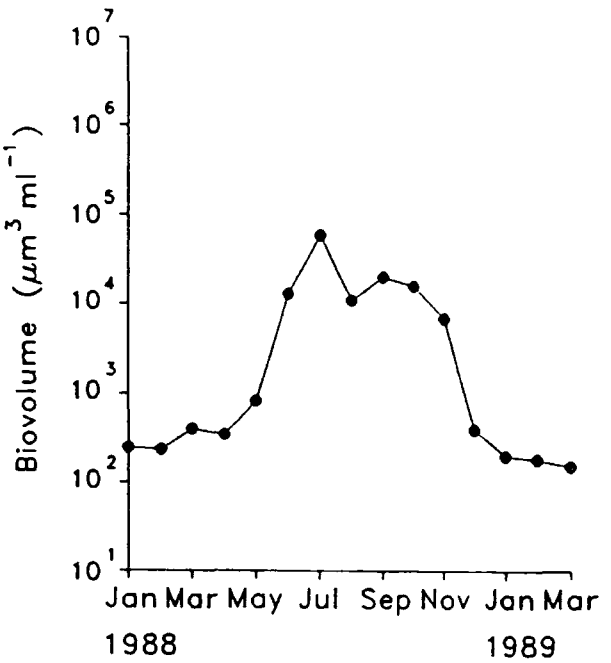


Malham Tarn

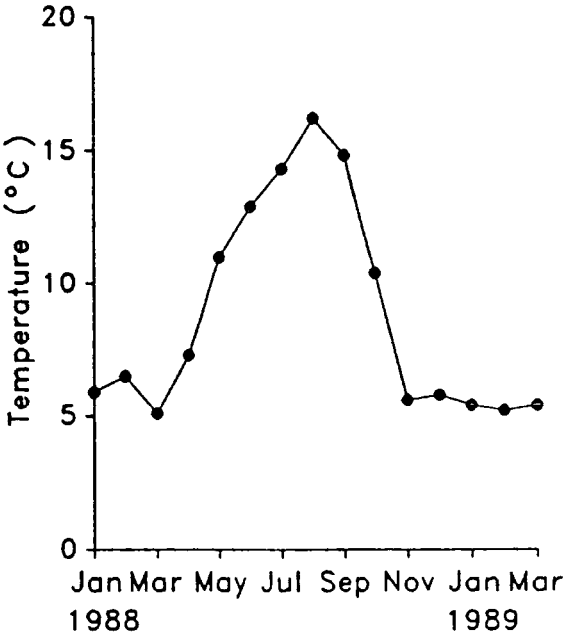

Temperature (°C)
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan Mar
1988
1989


Chl a (μg l⁻¹)
30
25
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan
1988
1989


Density (cells ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989


Biovolume (μm³ ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

Thirlmere


Temperature (°C)
20
15
10
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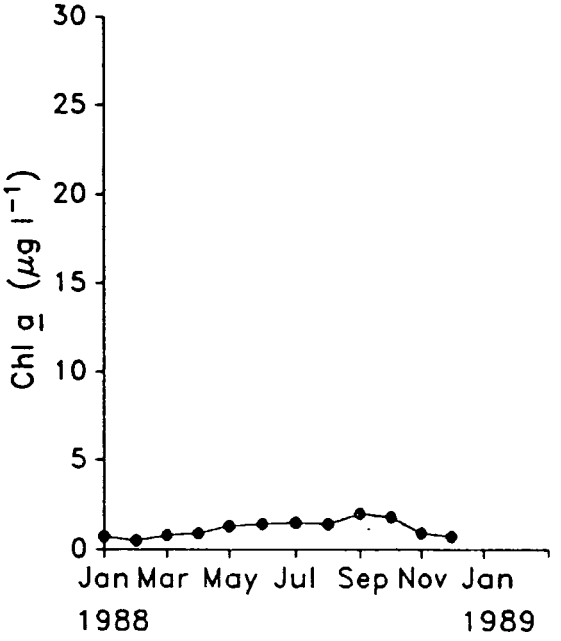
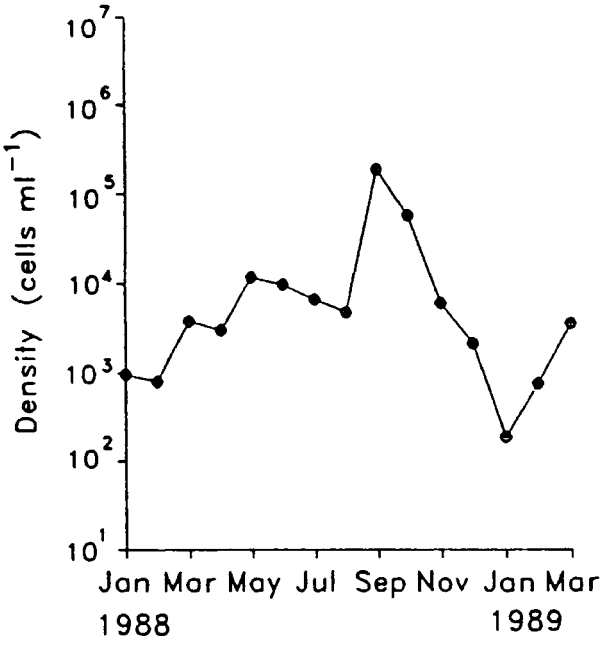
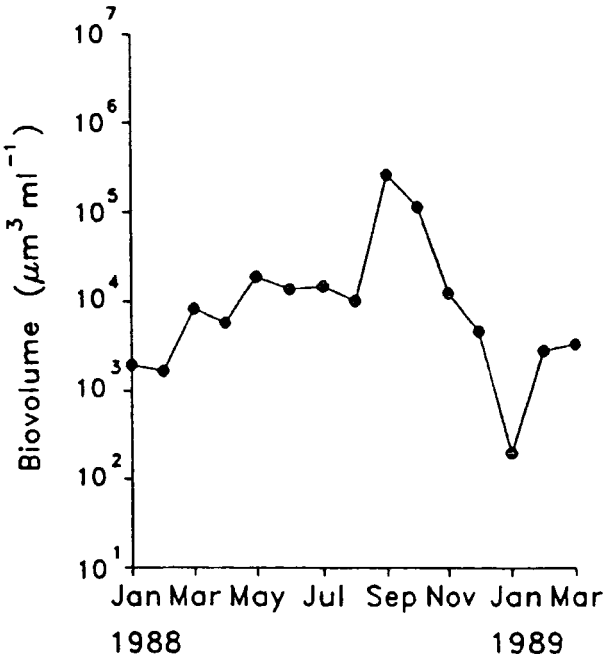
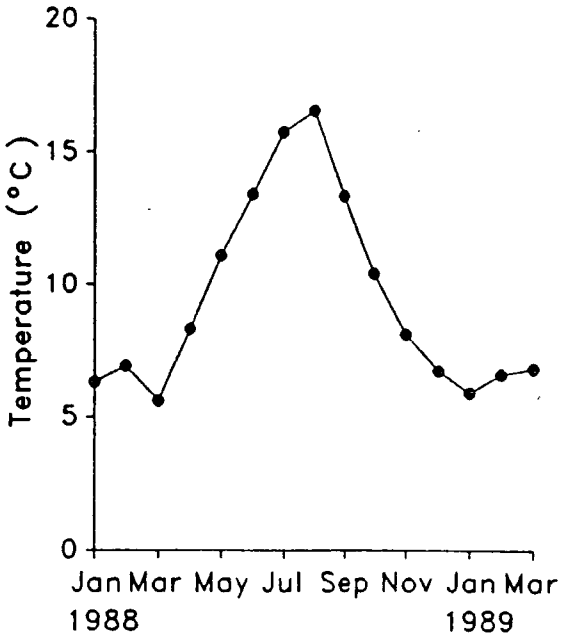
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

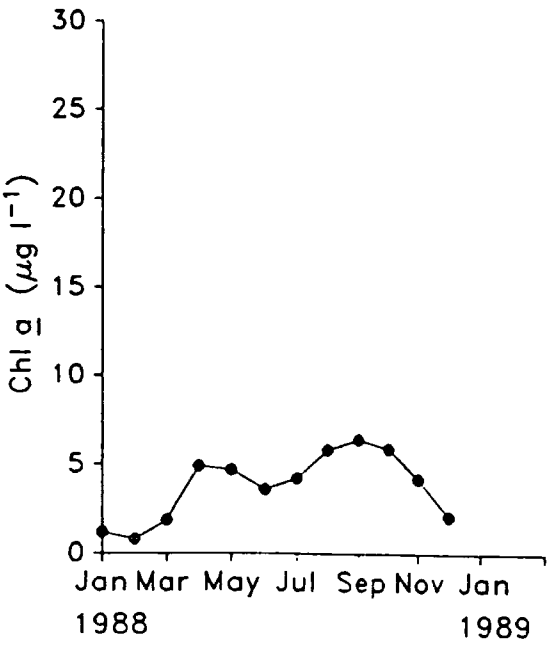

Chl a (μg l⁻¹)
30
25
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan
1988
1989

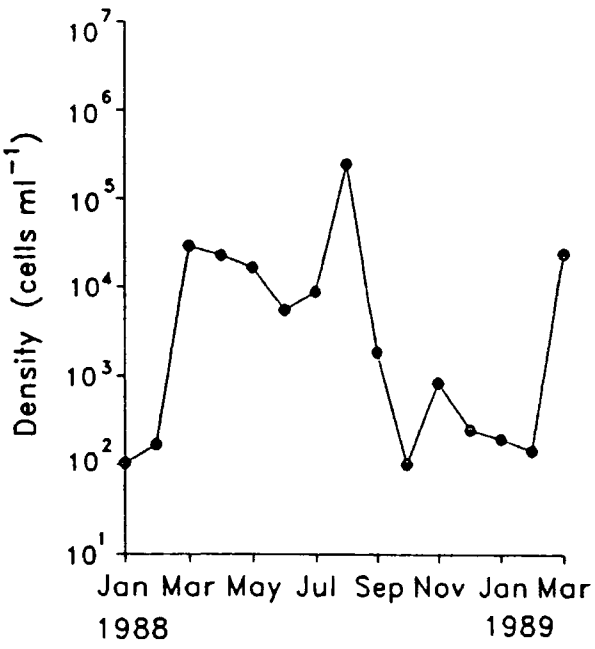

Density (cells ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

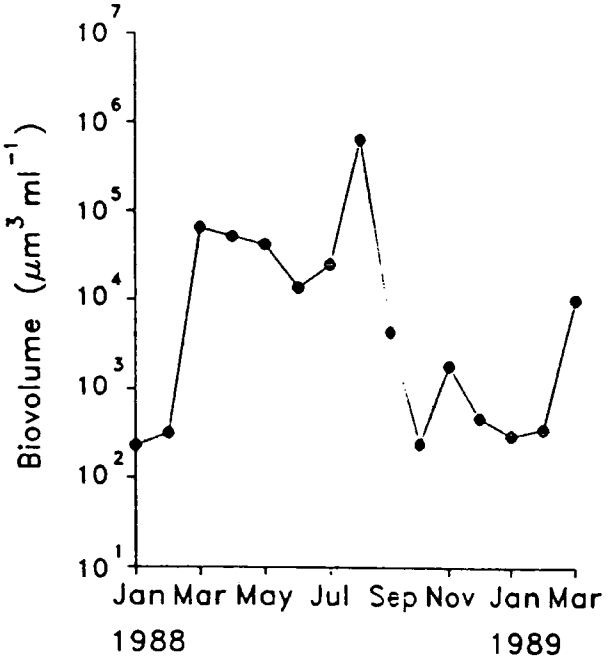

Biovolume (μm³ ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

Ullswater

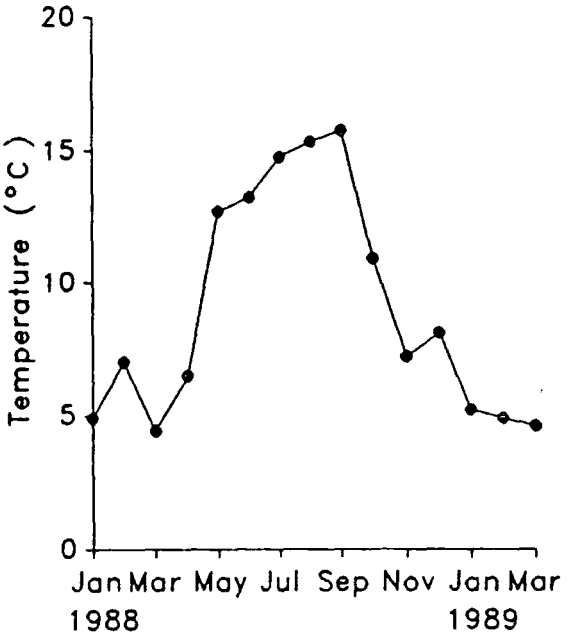

Temperature (°C)
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

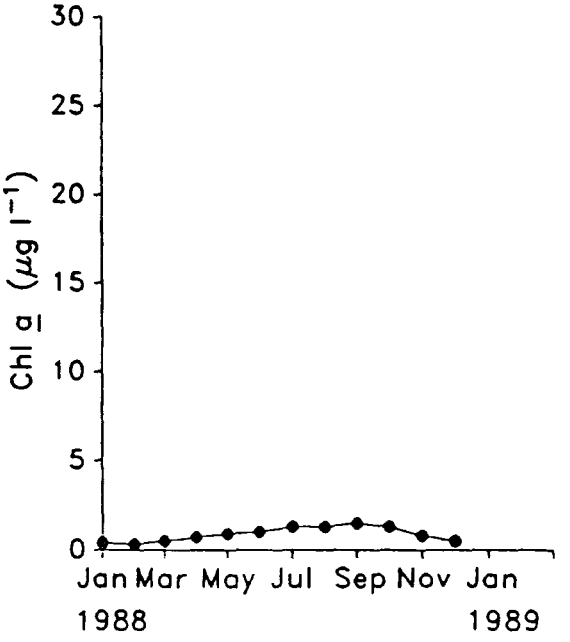

Chl a (μg l⁻¹)
30
25
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan
1988
1989

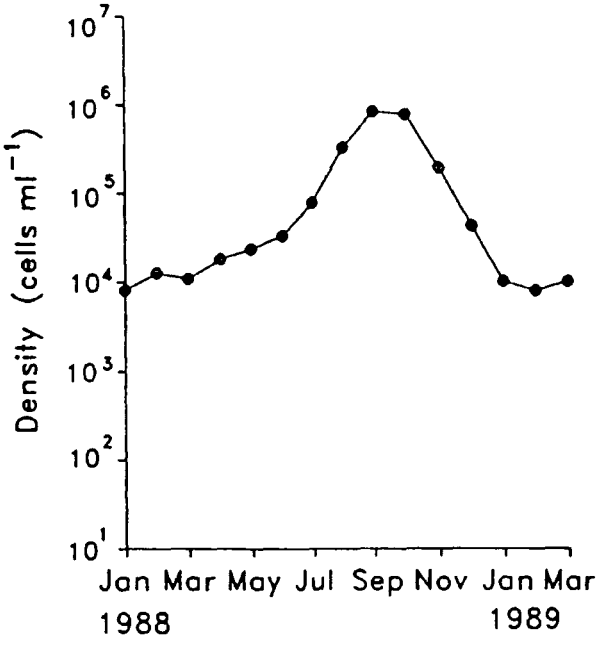

Density (cells ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

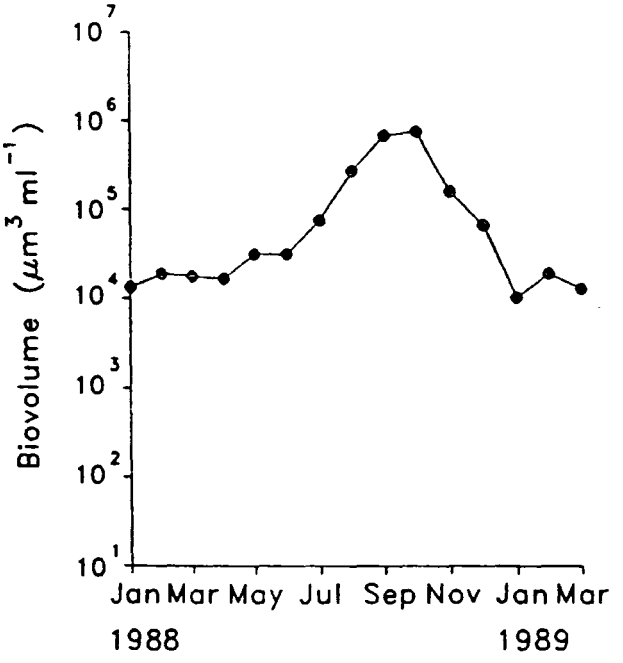

Biovolume (μm³ ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

Wastwater

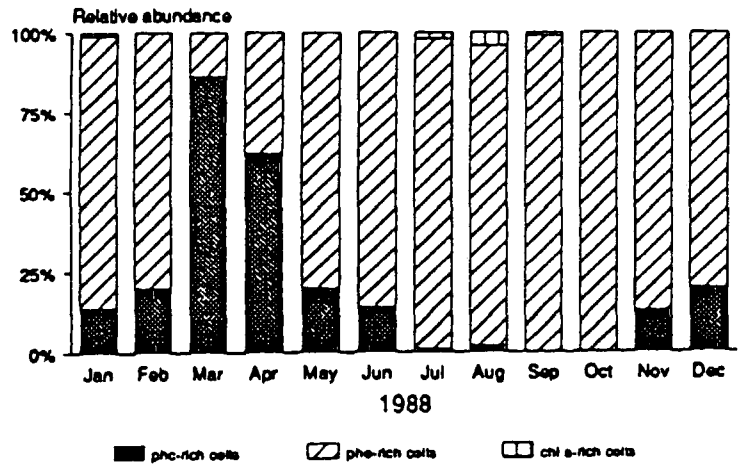

Temperature (°C)
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan Mar
1988
1989


Chl a (μg l⁻¹)
30
25
20
15
10
5
0
Jan Mar May Jul Sep Nov Jan
1988
1989

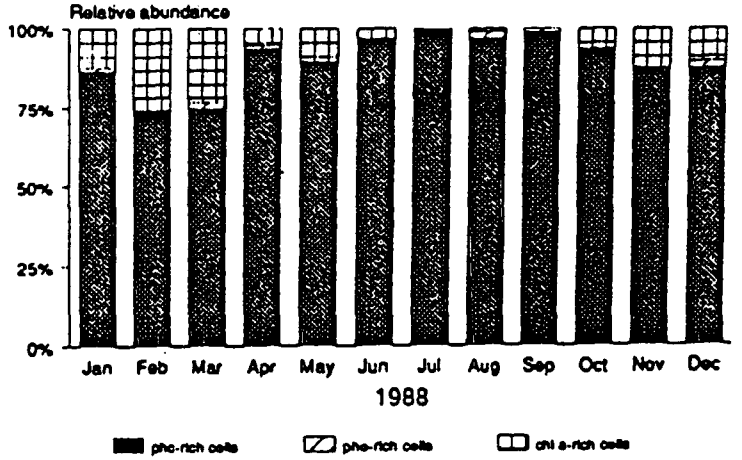

Density (cells ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989


Biovolume (μm³ ml⁻¹)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Jan Mar May Jul Sep Nov Jan Mar
1988
1989

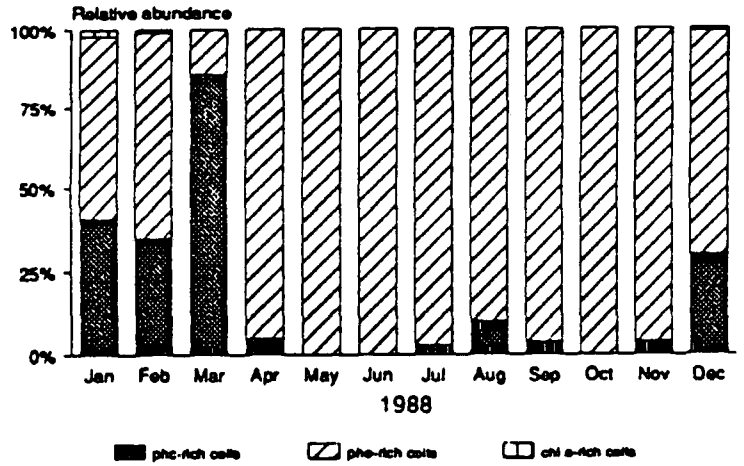
Bassenthwaite Lake



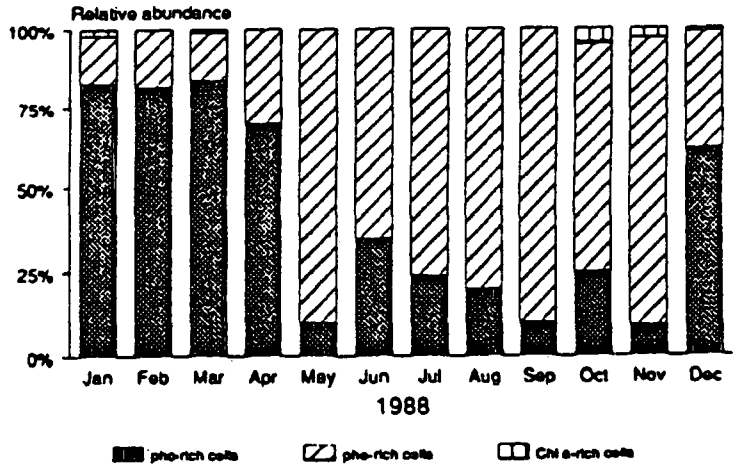
Cassop Pond



Coniston Water



Derwentwater



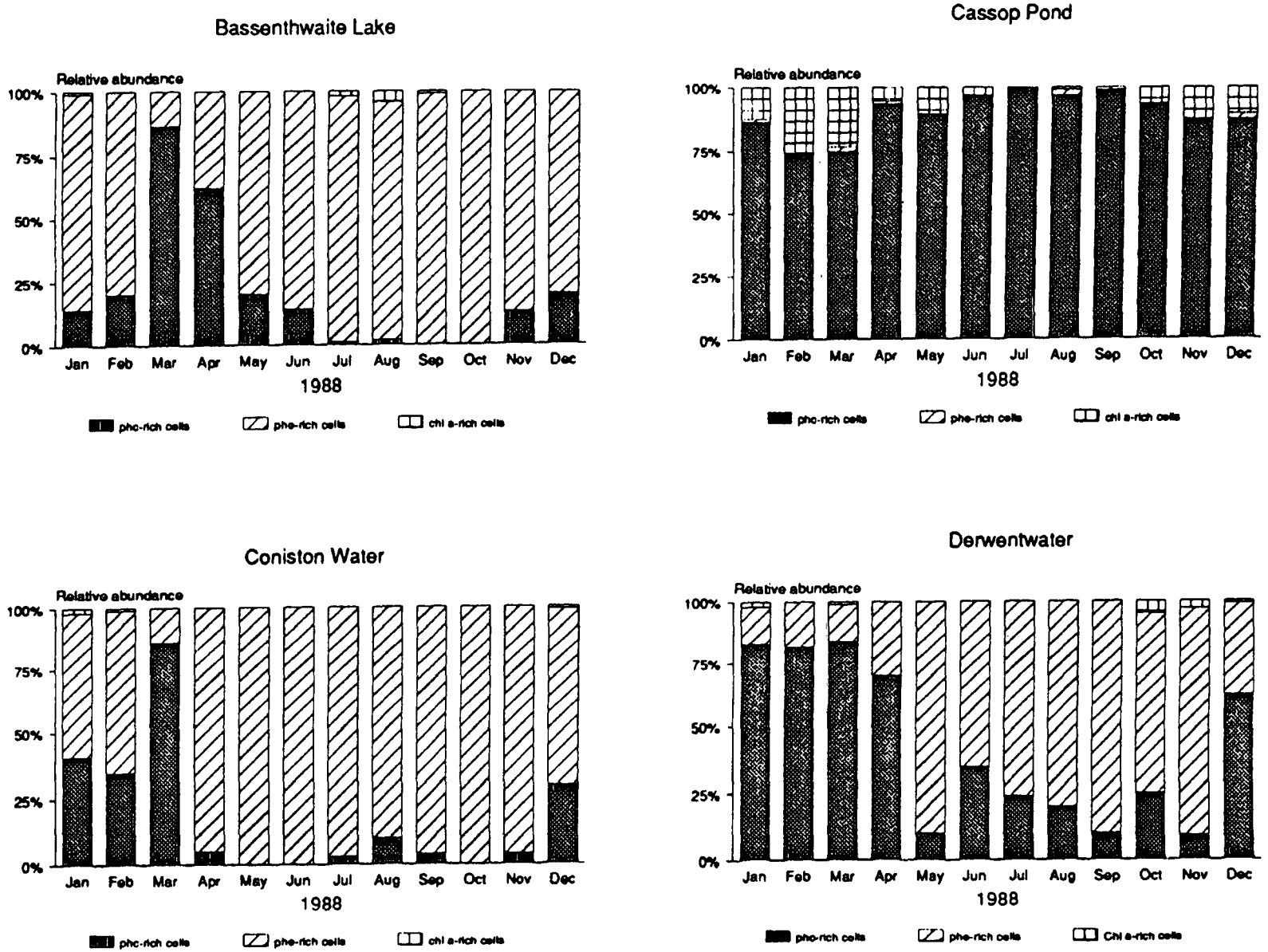
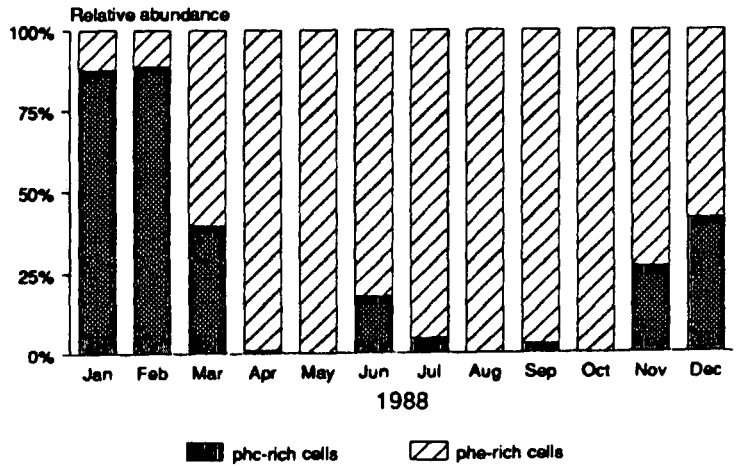
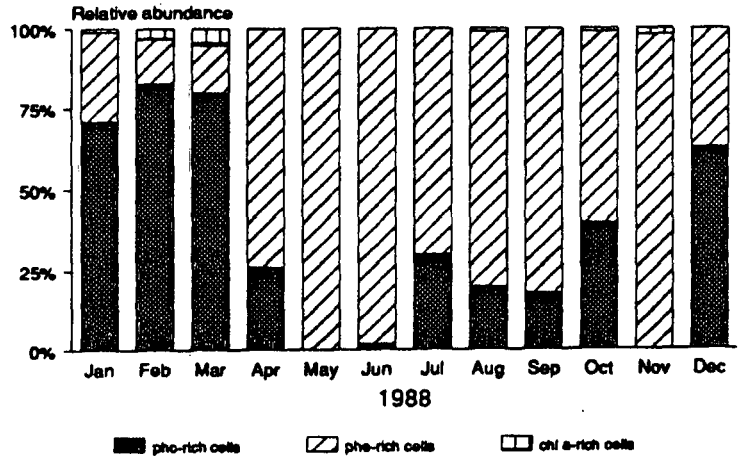


Fig 5.3 Relative abundance of phycocyanin-rich, phycoerythrin-rich and chlorophyll a-rich cells in ten UK lakes

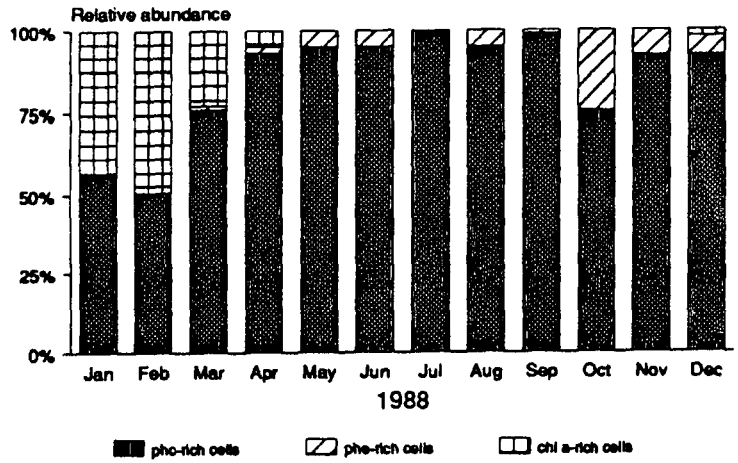
Ennerdale Water



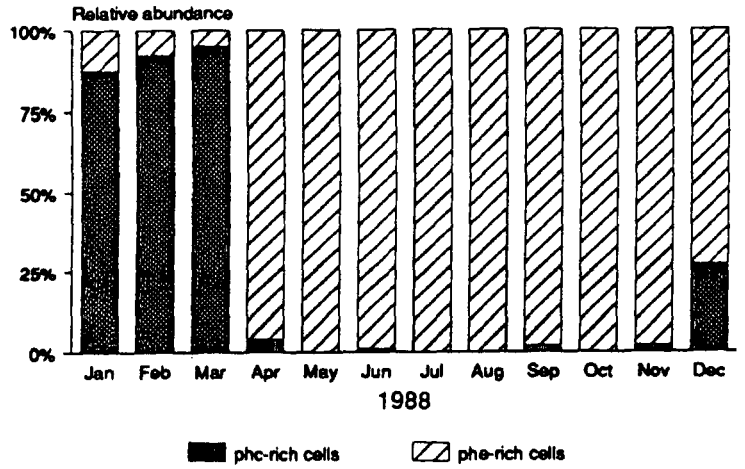
Esthwaite Water

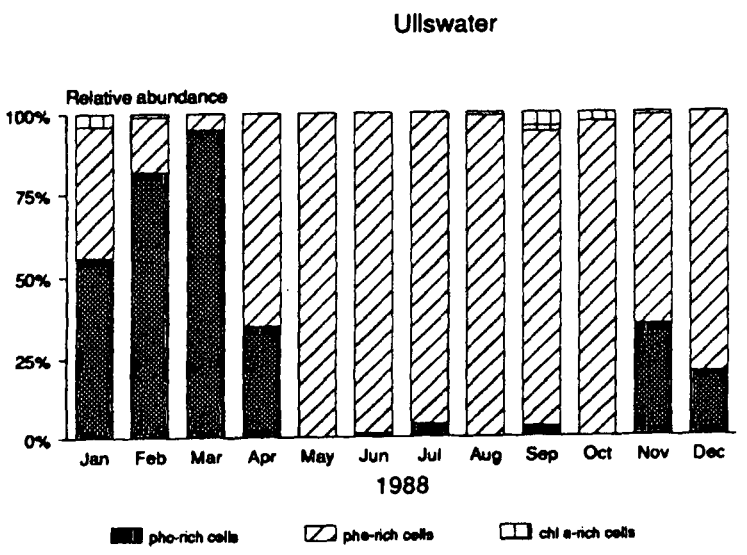


Malham Tarn



Thirlmere




Ullswater
Relative abundance
100%
75%
50%
25%
0%
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
1988
phc-rich cells
phe-rich cells
chl a-rich cells

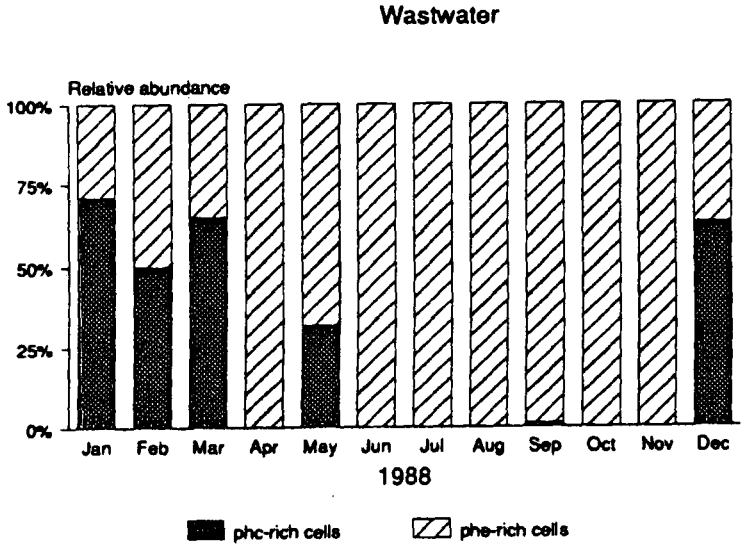

Wastwater
Relative abundance
100%
75%
50%
25%
0%
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
1988
phc-rich cells
phe-rich cells

Table 5.3 Selected data for individual lakes during 1988, showing ranges for total chlorophyll a and autotrophic picoplankton cell density, together with values for the maximum : minimum autotrophic picoplankton cell densities. The data are presented in order of values for maximum total chlorophyll a ("trophic status"), and values (rank) are given for the ten lakes according to maximum autotrophic picoplankton density recorded (highest = 1)

	chl a ($mg\ m^{-3}$)			pico cell density (cells ml-1)			rank
	min	max	max/min	min	max	max/min	
Cassop Pond	9.8	30.0	3.0	1.22×10^2	1.65×10^4	135	9
Malham Tarn	1.6	26.4	16.5	1.18×10^2	3.26×10^4	276	8
Esthwaite	2.0	20.2	10.1	8.78×10^1	3.42×10^3	39	10
Bassenthwaite	2.3	19.4	8.4	7.01×10^2	7.92×10^4	112	7
Coniston	1.5	9.3	6.2	2.35×10^2	4.50×10^5	191	3
Derwentwater	1.1	7.6	6.9	7.44×10^2	2.65×10^5	356	4
Ullswater	0.8	6.4	8.0	1.04×10^2	2.46×10^5	2360	5
Ennerdale	0.5	2.0	4.0	1.62×10^4	1.30×10^6	80	1
Thirlmere	0.6	1.8	3.0	9.56×10^2	1.91×10^5	199	6
Wastwater	0.3	1.5	5.0	8.04×10^1	8.21×10^5	102	2

All lakes showed temporal variation with respect to both autotrophic picoplankton density and relative abundance of cell type. Maximum densities were recorded in Wastwater in October with 1.06×10^6 cells ml^{-1}. Lowest densities were in Esthwaite Water in May (1.15×10^2 cells ml^{-1}). All showed a range of total autotrophic picoplankton density (all fluorescing types) of at least two orders of magnitude within an annual cycle. The maximum range was recorded in Ullswater with a difference between summer and winter densities of more than three orders of magnitude. The least seasonal difference was recorded in Wastwater with a range of two

orders of magnitude.

All but one of the lakes (Bassenthwaite Lake) showed a maximum for total chlorophyll a in mid- to late summer and all the lakes showed a maximum for autotrophic picoplankton density in mid- to late summer, though not necessarily at the same time as total chlorophyll. However, Esthwaite also showed an obvious spring peak in autotrophic picoplankton cell density. The maximum values for autotrophic picoplankton occurred in July (1 lake), August (4 lakes), September (3 lakes) and October (2 lakes). Overall autotrophic picoplankton density ranged during the year over about one order of magnitude more than total chlorophyll a (Table 5.3).

The proportion of phycoerythrin-rich autotrophic picoplankton to total autotrophic picoplankton showed a minimum in winter and a maximum in summer in all the lakes, though the percentage values for the two shallow lakes (Cassop Pond and Malham Tarn) were always very low (Table 5.4). The percentage values showed a marked increase in all the lakes of the English Lake District some time between March and May and a decrease in December.

Table 5.4 Comparison of mean percentage of phycoerythrin (PE)- rich cells and autotrophic picoplankton cell volume for the months when autotrophic picoplankton cell density is minimum and maximum. The data are presented in order of values for maximum total chlorophyll a ("trophic status").

	when cell density is min.		when cell density is max.	
	mean vol. (μm^3)	PE-rich cells (%)	mean vol. (μm^3)	PE-rich cells (%)
Cassop Pond	1.89	1	2.04	2
Malham Tarn	2.10	0	1.77	5
Esthwaite	1.90	14	2.38	59
Bassenthwaite	1.30	80	1.33	94
Coniston	1.74	64	1.75	90
Derwentwater	1.93	18	1.23	70
Ullswater	1.67	40	2.62	99
Ennerdale	1.34	12	0.87	97
Thirlmere	2.08	8	1.37	98
Wastwater	1.66	29	0.82	100

The range of autotrophic picoplankton biovolume for the ten lakes at the time of minimum autotrophic picoplankton density was less (1.32 - 2.10 μm^3) than at the time of the maximum picoplankton density (0.87 - 2.62 μm^3) (Table 5.5). Four of the five most oligotrophic lakes showed a marked decrease in mean autotrophic picoplankton cell volume in summer, but one (Ullswater) showed an increase.

Table 5.5 Summary of data for phytoplankton in the ten lakes, including ranges of values for January and for the months when minimum and maximum values for autotrophic picoplankton cell density occur for any particular lake. The final column shows the ratio of maximum to minimum values.

	range	unit	ratio
total chl a, Jan	0.4 - 13.1	$mg\ m^{-3}$	32
total chl a, minimum	0.3 - 9.8	$mg\ m^{-3}$	32
total chl a, maximum	1.5 - 30.0	$mg\ m^{-3}$	20
picoplankton density, Jan	1.00×10^2 - 1.62×10^4	cells ml^{-1}	162
picoplankton density, min.	4.20×10^3 - 1.62×10^4	cells ml^{-1}	382
picoplankton density, max.	3.12×10^3 - 1.30×10^6	cells ml^{-1}	416
mean pico-cell vol., Jan	1.30 - 2.21	μm^3	1.7
mean pico-cell vol. when density min.	1.32 - 2.10	μm^3	1.6
mean pico-cell vol. when density max.	0.87 - 2.62	μm^3	3.0
picoplankton biovolume,Jan	1.90×10^2 - 2.18×104	$\mu m^3\ ml^{-1}$	114
picoplankton biovolume,min	1.82×10^2 - 2.18×104	$\mu m^3\ ml^{-1}$	119
picoplankton biovolume,max	8.14×10^3 - 1.14×106	$\mu m^3\ ml^{-1}$	140

In all but one lake cell densities and biovolumes for the period January - March 1989 were very similar to those for the same period in 1988. The exception was Thirlmere, where the January 1988 value for density and mean biovolume was much higher than the 1989 value (10^3 v 1.8×10^2 cells ml^{-1}, 2.8×10^3 v 2.6×10^2 $m^3\ ml^{-1}$).

Correlation coefficients of autotrophic picoplankton density with temperature and total chlorophyll were calculated. The coefficients are presented in Table 5.6.

Table 5.6 Correlation coefficients of autotrophic picoplankton density and temperature and total chlorophyll a in ten lakes. Data sets for the whole of the seasonal study were used in the calculations. The coefficients are ranked according to the strongest correlation (highest = 1).

	$\log_{10}$ picoplankton density v temp (n = 15)	Rank	$\log_{10}$ picoplankton density v chl a (n = 12)	Rank
Bassenthwaite	+0.500	7	+0.400	8
Cassop	+0.941	1	+0.676	6
Coniston	+0.853	3	+0.834	3
Derwentwater	+0.548	6	+0.911	1
Ennerdale	+0.667	5	+0.616	7
Esthwaite	+0.304	10	+0.732	4
Malham Tarn	+0.930	2	+0.689	5
Thirlmere	+0.444	9	+0.239	10
Ullswater	+0.500	7	+0.391	9
Wastwater	+0.728	4	+0.839	2

In many of the lakes $\log_{10}$ autotrophic picoplankton density are only weakly positively correlated with chlorophyll a or temperature. Notable strong correlations are $\log_{10}$ autotrophic picoplankton density and temperature in Cassop Pond, $\log_{10}$ autotrophic picoplankton density and temperature in Malham Tarn and $\log_{10}$ autotrophic picoplankton density and chlorophyll a in Derwentwater. The strong density and temperature correlations occurred in the two most shallow lakes, where the water did not stratify in the summer months.

apparent.

Table 5.7 Net doubling time of autotrophic picoplankton populations in the spring and summer period. The period corresponded to the spring population increase to the summer maximum which was different from lake to lake.

	Period used for calculation	days / division
Bassenthwaite Lake	May - Aug	14.6
Cassop Pond	Apr - Aug	18.1
Coniston Water	Feb - Aug	18.2
Derwentwater	Mar - Oct	47.6
Ennerdale Water	May - Sep	22.2
Esthwaite Water	May - Oct	22.0
Malham Tarn	Apr - Jul	10.3
Thirlmere	Apr - Sep	39.4

Ullswater	Feb - Aug	30.0
Wastwater	Mar - Sep	27.3

The minimum division time was over a week (Malham Tarn) and the maximum nearly seven weeks (Derwentwater). This does not represent the true doubling time of cells as the impact of grazers and other losses are unknown; nevertheless, it provides a minimum growth rate during the warming period. In all the lakes studied, the autotrophic picoplankton growing season extended over several months; typically the autotrophic picoplankton population increased steadily from early summer and reached a maximum in late summer.

5.3 AUTOTROPHIC PICOPLANKTON DENSITIES IN MICROHABITATS

5.31 Study sites

Three sites were selected to test whether there were differences in autotrophic picoplankton density in a number of microhabitats: Cassop Pond, Co. Durham, Ennerdale Water, Cumbria and Surha Tal deepwater rice field in Uttar Pradesh, India. These sites were regarded as meso-eutrophic, oligotrophic and mesotrophic, respectively, based on analysis of total filtrable phosphorus analyses (Section 5.22). Phosphorus analysis was not carried out at Surha Tal, but the environment is similar to deepwater rice fields in Bangladesh which are described as mesotrophic (Whitton et al., 1988).

5.32 Method

Four replicates were taken from 0.5 m depth at eight sites at two waterbodies and seven sites at one waterbody; replicates were collected

within approximately one minute of each other. Samples from Cassop Pond were collected in May 1988, from Ennerdale Water in June 1988 and from Surha Tal in September 1988. Samples were preserved and counted in the laboratory at Durham. Coefficients of variation were calculated according to the formula in Section 2.593.

5.33 Results

The results are presented in Tables 5.8 - 5.9

Table 5.8 Microhabitat distribution of autotrophic picoplankton cells in Cassop Pond

Site	Microhabitat	Density cells ml^{-1}	CV (%) n=4
1	Typha fringe	$3.42 \times 10^3 \pm 723$	21.1
2	Typha fringe	$1.20 \times 10^2 \pm 80$	66.7
3	Potamogeton fringe	$4.14 \times 10^3 \pm 103$	24.9
4	Embayment	$3.29 \times 10^3 \pm 419$	12.7
5	Open water	$7.46 \times 10^3 \pm 732$	9.8
6	Open water	$5.23 \times 10^3 \pm 487$	9.3
7	Open water	$8.71 \times 10^2 \pm 111$	12.7
8	Open water	$1.03 \times 10^3 \pm 98$	9.5

Largest coefficients of variation were found from samples collected in the fringes of the pond which supported dense stands of Typha and Potamogeton. The mean coefficient of variation at the 5 open water sites ($\bar{x}$=10.8, n=5) was more than three times lower than the mean coefficient of variation in samples from the margins ($\bar{x}$=37.5, n=3)

Table 5.9 (a) Microhabitat distribution of autotrophic picoplankton in Ennerdale Water

Site	Microhabitat	Density cells ml^{-1} (± SD)	CV (%)
1	Open water	8.27×10^4 ±10050	12.2
2	Open water	6.11×10^4 ± 8600	14.1
3	Open water	3.23×10^5 ±42300	13.1
4	Open water	4.21×10^4 ± 1020	2.4
5	Open water	1.04×10^4 ± 5310	51.0
6	Rocky outcrop (submerged)	8.72×10^3 ± 1520	17.4
7	Shoreline	1.65×10^4 ± 4260	25.8
8	Shoreline	3.23×10^4 ± 7350	22.8

As at Cassop Pond largest coefficients of variation in Ennerdale Water occurred around the shore or at a rocky outcrop (although the maximum coefficient of variation is found at open water site 5). The mean coefficient of variation in the open water sites ($\bar{x}$=18.5, n=5) was not very much lower than mean coefficient of variation from the marginal sites ($\bar{x}$=22, n=3).

Table 5.9 (b) Microhabit distribution of autotrophic picoplankton in Surha Tal rice fields. (DWR = Deepwater rice)

Site	Microhabitat	Density cells ml^{-1} (± SD)	% PC-rich cells	CV (%)
1	Dense DWR	3.20×10^3 ±480	100	15.0
2	Dense DWR	3.13×10^3 ±524	100	16.7
3	Dense DWR	8.52×10^2 ±386	98	45.3
4	Open area	4.20×10^3 ±462	85	11.0
5	Open area	1.04×10^2 ± 42	96	40.4
6	Weedy DWR	5.32×10^2 ± 78	100	14.7
7	Weedy DWR	8.90×10^2 ± 43	94	4.8

At Surha Tal there were three categories of sample site (unlike Cassop Pond and Ennerdale Water where sampling sites were either open water or marginal microhabitats): Dense deepwater rice, where the tiller density was approximately 100 m^{-2}; weedy deepwater rice areas, where tiller density was

approximately 20 m^{-2}; and open areas, which were fallow areas and supported dense stands of aquatic macrophytes (particularly Ceratophyllum, Myriophyllum and Salvinia). Coefficients of varaition in samples from dense DWR ($\bar{x}$=25.6, n=3) and open areas ($\bar{x}$=25.7, n=2) were similar and the lowest coefficent of varaition were from samples in weedy DWR areas ($\bar{x}$=9.7, n=2).

5.4 VERTICAL DISTRIBUTION OF AUTOTROPHIC PICOPLANKTON

5.41 Introduction

Vertical profiles were taken on two dates to determine autotrophic picoplankton population distribution throughout the water column. The dates chosen aimed to coincide with maximum picoplankton densities in the reservoir; these were assumed from the temporal studies carried out in the ten lakes (Section 5.2) in which all the lakes studied had autotrophic picoplankton maxima towards the end of the summer.

5.42 Study site

Kielder Water was chosen for this study as the author was carrying out work on this site for a survey for the then Northumbrian Water Authority (Hawley & Whitton, unpublished report).

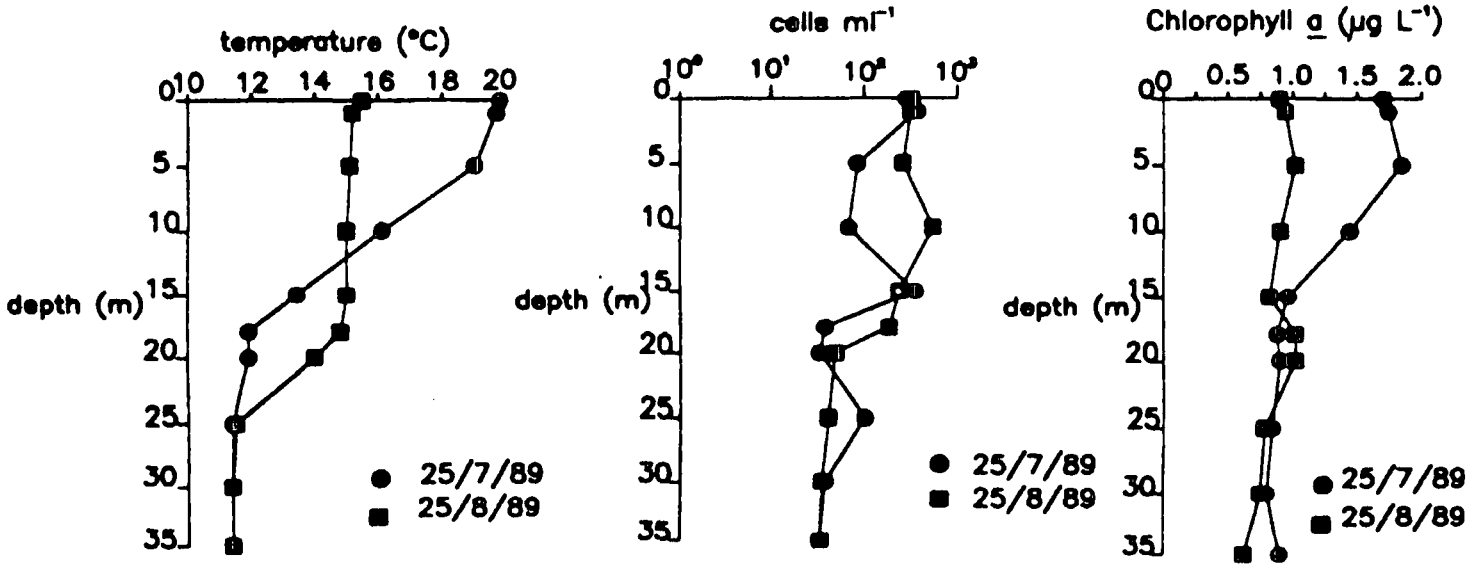
5.43 Results

The results of the vertical profiles are presented in Fig. 5.4. The first graph shows that the reservoirs were thermally stratified on both sampling occasions, with the epilimnion deepening by about 15 m between the two sampling dates. Autotrophic picoplankton densities were generally low compared with densities collected in other UK lakes and from samples

collected abroad; the maximum cell density from any depth on either sampling occasion was 7.3×10^3 cells ml^{-1}. Autotrophic picoplankton were present in all the samples analysed from below the thermocline, though densities were only just above the detection limit.

There was no notable trend in autotrophic picoplankton densities with increasing depth on the first sampling occassion, but by the following month autotrophic picoplankton densities in the epilimnion were about an order of magnitude higher than in the hypolimnion. Chlorophyll *a* concentrations on both dates were low. The concentration in the epilimnion was higher in July than August and the concentration in the hypolimnion was similar on both dates.

Fig 5.4 Vertical distribution of temperature, autotrophic picoplankton cell density and chlorophyll a at Kielder Water



CHAPTER 6

IN SITU INCUBATIONS OF AUTOTROPHIC PICOPLANKTON

6.1 INTRODUCTION

The results from the studies in Chapter 5 indicate that maximum autotrophic picoplankton densities occurred in mid- to late summer. Temperature probably influences autotrophic picoplankton densities (Section 1.62), but the literature review highlighted the importance of grazers in natural environments (Section 1.91). Studies were carried out to determine in situ growth rates in the absence of grazers and establish whether grazers can be a major factor limiting autotrophic picoplankton density in nature.

6.2 STUDY SITES

Experiments were conducted at three sites: Bassenthwaite Lake, Cumbria; Surha Tal deepwater rice field, India; Lake Gangebal in Kashmir, India. The justification for the sites was largely logistical: all sites were chosen because of convenient bases close to the sites.

6.3 METHOD

At each site a bucket of lake water was taken and divided into two 2-litre aliquots. One aliquot was filtered through a 3-μm filter to remove grazers and the other aliquot was left unfiltered. Both aliquots were incubated in glass flasks standing in water at the edge of the lake. 20 ml samples were

removed every 6 h for 42 h (30 h at Gangebal), preserved and returned to Durham. Duplicates of each flask were incubated. Gangebal and Surha Tal incubations were carried out in September 1988 and the Bassenthwaite incubation in November 1988. The method assumed that grazers were larger than about 3 μm and therefore not part of the filtrate.

6.4 RESULTS

The results are presented in Fig. 6.1. Incubations at each site showed increased densities in filtered incubations compared with the control. Cell densities in Lake Gangebal incubations dropped markedly after 24 h.

6.41 Maximum growth rates in incubations

Maximum growth rates were calculated for the period with the greatest increase in cell density (assessed from the curves by eye) using the formula in Section 2.592. The results are presented in Table 6.1.

Fig. 6.1 In situ incubations of 3-μm filtrates in Bassenthwaite Lake, Surha Tal, and Lake Gangebal. In each graph the top line is the 3-μm-filtered incubation and the bottom line, the unfiltered control. Note the y-axis scale is different on the graphs. Each point is the mean of two duplicates.

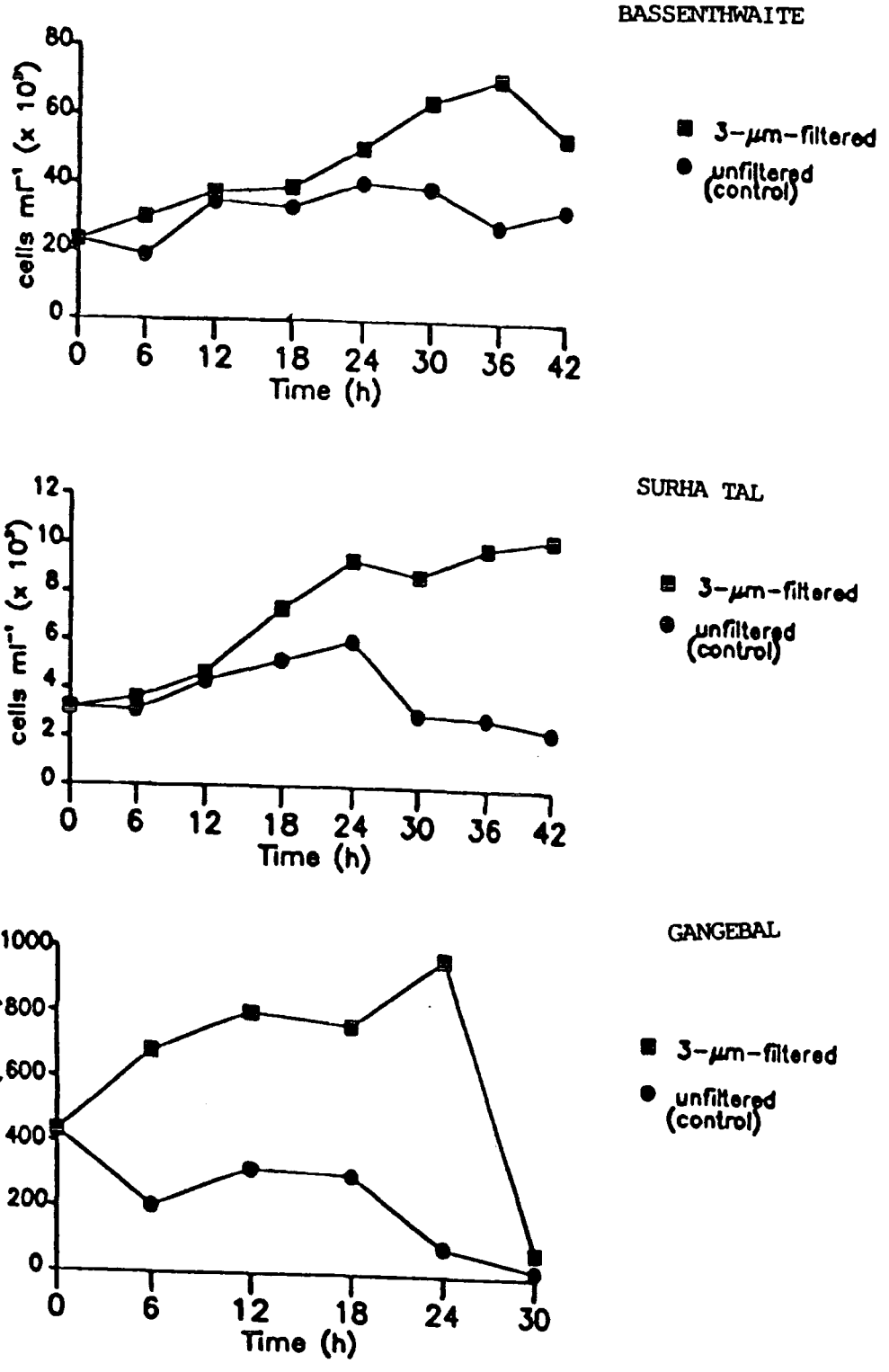


Table 6.1 Minimum division times of autotrophic picoplankton cells in 3-μm filtered incubations.

Waterbody	t_d (h)
Bassenthwaite Lake	16.4
Surha Tal	11.0
Gangebal	12.0

Autotrophic picoplankton at Surha Tal had the shortest division time and autotrophic picoplankton had lower division times in both the experiments at sub-tropical sites compared with the temperate site.

6.5 DISCUSSION

The results suggest that grazers played a major role in the control of the autotrophic picoplankton population at the sites. The minimum division times in the control (unfiltered) incubation at each of the three sites was considerably longer than filtered incubations, supporting the hypothesis that grazers are important controllers of autotrophic picoplankton populations.

CHAPTER 7

PHYSIOLOGICAL COMPARISON OF STRAINS

7.1 INTRODUCTION

Chapters 4, 5 and 6 concerned autotrophic picoplankton populations in natural environments. Epifluorescence microscopy enabled phycocyanin-rich and phycoerythrin-rich cells to be distinguished from each other, but little other information could be gleaned from microscopy. The aim of the physiological investigations in this chapter was to determine whether or not strains were similar with respect to growth under different conditions. To achieve this, a number of strains were isolated in clonal axenic culture (Section 2.72) and used in laboratory studies. Studies focussed on the utilization of nitrogen and phosphorus substrates as they are generally regarded as growth-limiting.

7.2 PICOPLANKTON STRAINS

Strain origin and history are presented in Section 2.8.

7.3 EFFECT OF NITROGEN SOURCES AND LIGHT ON GROWTH

Experiments were conducted to determine whether or not strains showed different growth rates when incubated with ammonium nitrogen (NH_4-N), nitrate nitrogen (NO_3-N) or urea nitrogen (urea-N) in Chu 10 medium. Further

experiments investigated growth rates of strains grown under different light flux.

7.31 Method

Strains were incubated in duplicate boiling tubes in a shaking water bath at 32 °C and their population densities were recorded at 24 h intervals for 14 d. Results from preliminary experiments showed that some strains had long lag periods before reaching maximum growth rates and therefore growth experiments were conducted over a two week period to ensure maxima were detected.

Experiments investigating growth in nitrogen sources were conducted in Chu 10 medium (Section 2.52) with nitrogen sources (NH_4-N, NO_3-N, urea-N) added at a final concentration of 10 mg l^{-1}-N; the light flux was 100 μmol photon m^{-2} s^{-1}. Experiments investigating the effect of light flux on growth were conducted in Chu 10 medium with NH_4-N (10 mg l^{-1}) and light flux was reduced to 80 and 40 μmol photon m^{-2} s^{-1} by wrapping neutral density filters around the boiling tubes.

7.32 Results

The growth curves are presented in Fig. 7.1. Most strains showed a lag phase of about 24 h before growth commenced. The exceptions were D773 and D797 in NH_4-N, which did not have a lag phase, although in D773 growth slowed after 24 h for a period of 24 h. Lag phases were often longer in incubations with urea-N (eg D767, D768, D773). In 8 strains the final yield was approximately 10^8 cells ml^{-1} in all the nitrogen sources. The final yield in all nitrogen sources in D562 was about an order of magnitude lower

than this and in D773 a yield of 10^8 cells ml^{-1} was reached in the NH_4-N incubation but only 10^7 cells ml^{-1} in the other nitrogen substrates.

In most strains, growth was fastest in NH_4-N or NO_3-N in the 24 h period following the lag phase. The exceptions were D562, in which strains incubated in NH4-N grew fastest between 192 and 240 h.

In general the yield (cells ml^{-1}) was greatest in incubations grown in 100 μmol photon m^{-2} s^{-1} compared with incubations in lower light flux and invariably, yields were lowest in 40 μmol photon m^{-2} s^{-1}.

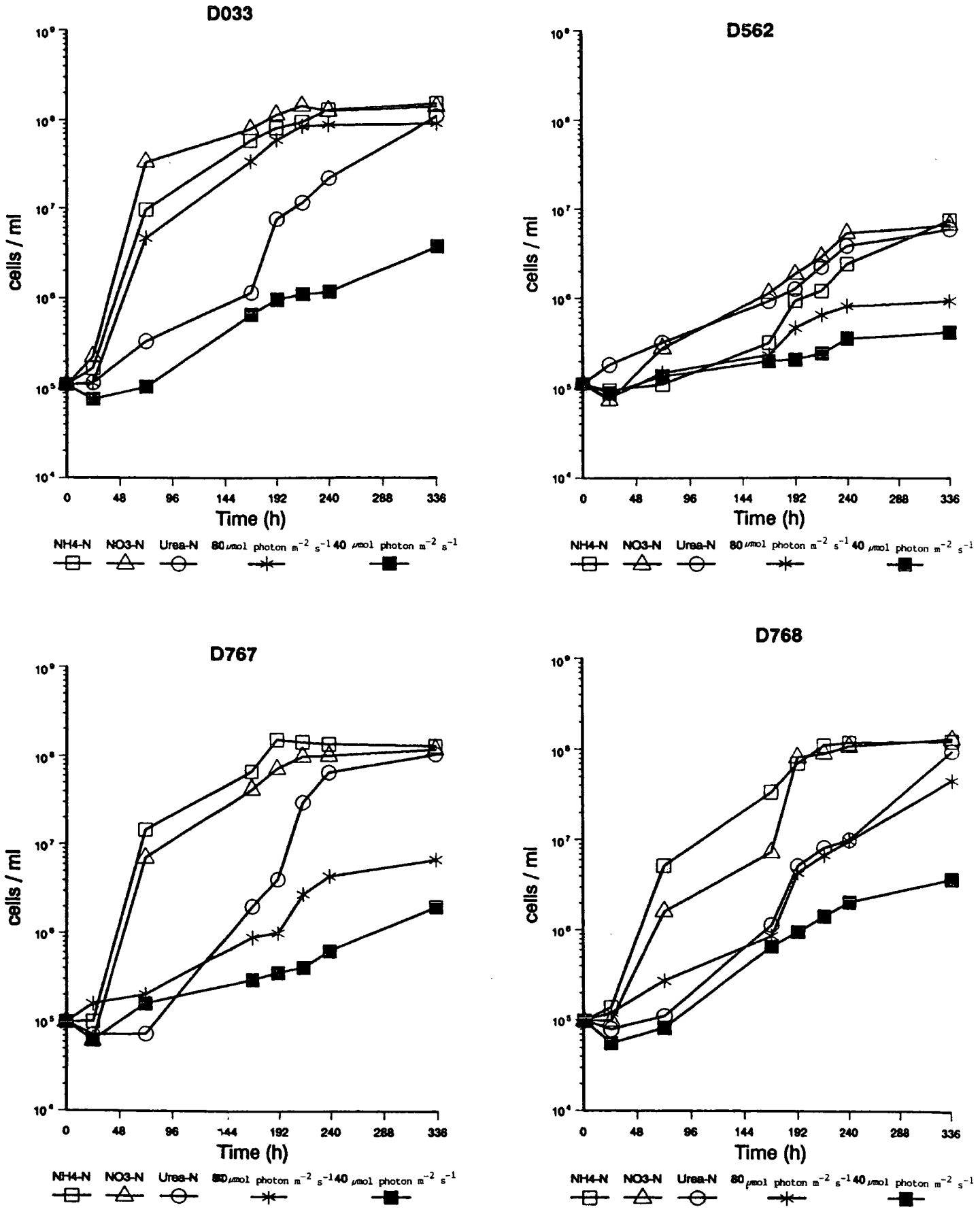
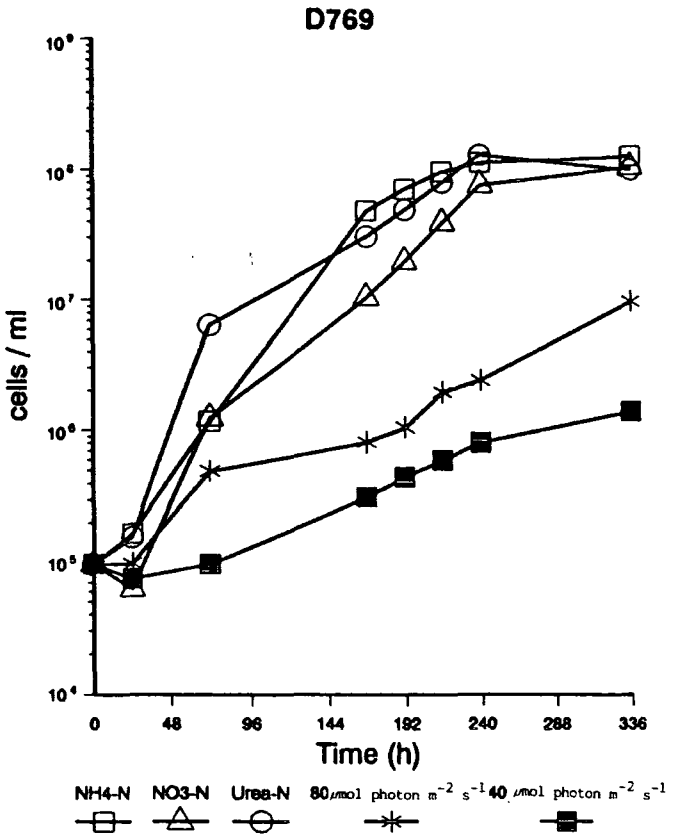
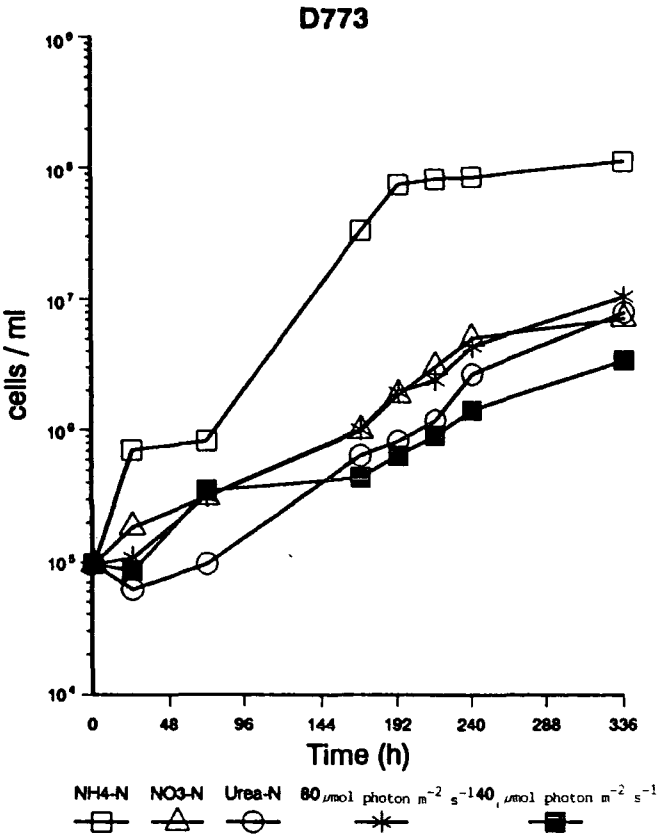
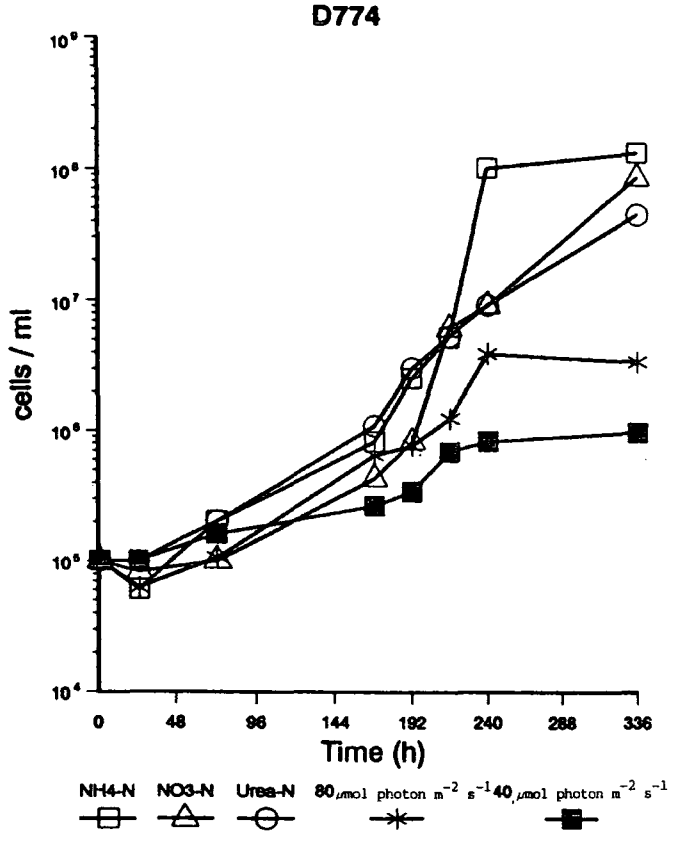
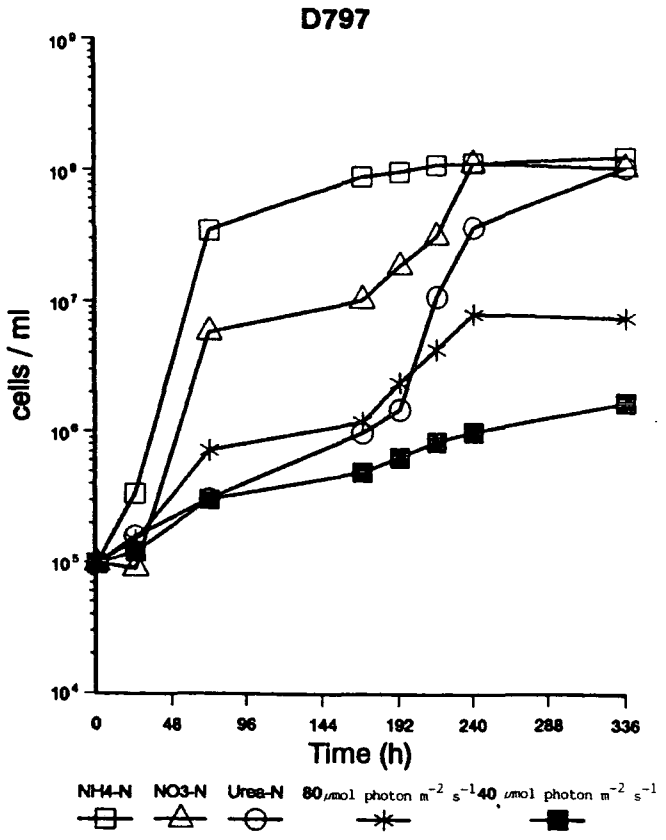


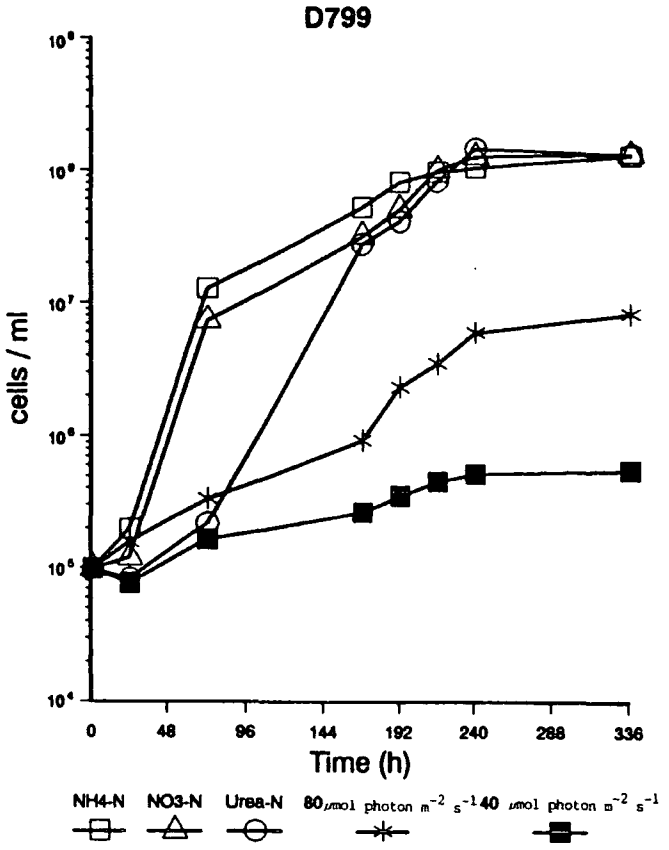
Fig 7.1 Growth curves of autotrophic picoplankton strains grown at 32 °C in Chu 10 medium with different nitrogen sources (all at 10 mg l^{-1} N) and in different light intensities. The experiments investigating growth in the different nitrogen substrates were conducted in 100 µmol photon m^{-2} s^{-1}. The experiments investigating light flux on growth were carried out in Chu 10 medium + NH_4-N (10 mg l^{-1}).

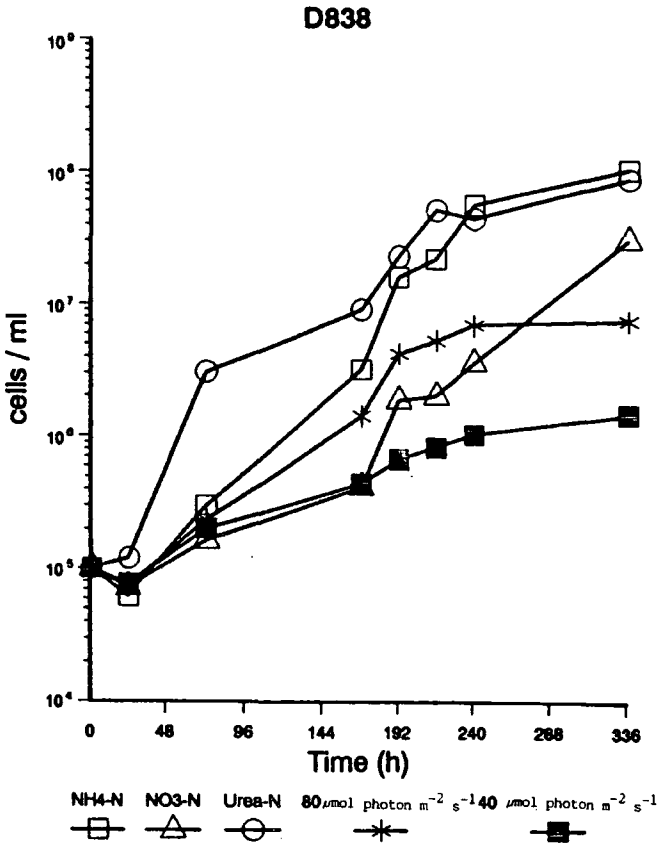

D769
cells / ml
Time (h)
0
48
96
144
192
240
288
336
NH4-N
NO3-N
Urea-N
80 µmol photon m-2 s-1
40 µmol photon m-2 s-1


D773
cells / ml
Time (h)
0
48
96
144
192
240
288
336
NH4-N
NO3-N
Urea-N
80 µmol photon m-2 s-1
40 µmol photon m-2 s-1


D774
cells / ml
Time (h)
0
48
96
144
192
240
288
336
NH4-N
NO3-N
Urea-N
80 µmol photon m-2 s-1
40 µmol photon m-2 s-1


D797
cells / ml
Time (h)
0
48
96
144
192
240
288
336
NH4-N
NO3-N
Urea-N
80 µmol photon m-2 s-1
40 µmol photon m-2 s-1


D799
cells / ml
Time (h)
0
48
96
144
192
240
288
336
NH4-N
NO3-N
Urea-N
80 µmol photon m-2 s-1
40 µmol photon m-2 s-1


D838
cells / ml
Time (h)
0
48
96
144
192
240
288
336
NH4-N
NO3-N
Urea-N
80 µmol photon m-2 s-1
40 µmol photon m-2 s-1

The fastest doubling time for each curve was calculated from the steepest part of the growth curve (Section 2.562). The results are presented in Table 7.1.

Table 7.1 Minimum doubling times (h) of strains grown in 3 nitrogen substrates and 3 light regimes. NH_4-N, NO_3-N and Urea-N incubations were carried out in 100 μmol photon m^{-2} s^{-1}. 80 and 40 μmol photon m^{-2} s^{-1} incubations were in Chu 10 media with NH_4-N (10 mg l^{-1}).

Strain	NH_4-N	NO_3-N	Urea-N	80 μmol photon m^{-2} s^{-1}	40 μmol photon m^{-2} s^{-1}
D033	5.67	5.64	14.28	5.83	14.97
D562	24.00	33.92	28.06	31.28	29.61
D767	5.12	5.10	12.00	22.51	21.34
D768	6.06	8.48	16.96	16.20	13.50
D769	5.87	6.50	6.32	38.23	52.54
D773	7.39	33.37	28.50	38.84	42.74
D774	18.08	13.85	30.29	20.50	37.92
D797	5.67	7.04	10.38	26.08	23.80
D799	6.83	5.97	6.36	25.30	62.55
D838	17.26	21.25	19.26	25.09	18.91

Six out of the 10 strains had minimum doubling times when grown in NH_4-N; the other 4 strains grew fastest in NO_3-N. The minimum doubling time of any strain was 5.1 h by D767 grown in NO_3-N. No strain grew fastest in urea-N, and in 5 strains growth was slower in this substrate than in NH_4-N or NO_3-N.

Table 7.2 Minimum doubling time of the ten strains in any of the growth conditions. Strains are ranked according to doubling times (fastest = 1) and their country of origin is listed.

Strain	Minimum doubling	Rank	Country of origin
D767	5.1	1	Bangladesh
D033	5.64	2	Nepal
D797	5.67	3	USA
D769	5.87	4	Bangladesh
D799	5.97	5	Bangladesh
D768	6.06	6	Bangladesh
D773	7.39	7	UK
D774	13.85	8	UK
D838	17.26	9	USA
D562	24.00	10	USA

Five of the top six strains with the fastest doubling times are from subtropical climates. The three strains with minimum doubling times over 10 h were all isolated from waters in temperate climates.

7.4 PHYSIOLOGICAL INVESTIGATIONS

7.41 Introduction

The response of strains were investigated to a number of physiological tests to determine whether the strains were similar to each other or not. Chemoheterotrophy, chromatic adaptation, nitrogen fixation, motility, desiccation tolerance and the ability to be cryopreserved were all tested.

7.42 Methods

Chemoheterotrophic growth was tested in a range of organic carbon sources (Section 2.63). Evidence for nitrogen fixation was determined using the acetylene reduction assay (Section 2.621). Chromatic adaptation was assessed by incubating cultures in different light quality provided by

coloured filters. Motility was assessed microscopically (Section 2.66). Desiccation tolerance was investigated by drying cultures on two substrates and determining whether or not cells were viable following re-wetting (Section 2.65). Cryopreservation studies examined viability after storage in liquid nitrogen for 6 months (Section 2.64).

7.43 Results

The results are presented in Table 7.3. Light and epifluorescence microscopy revealed some differences in shape and size between strains. Cells were either elliptical, rod-shaped or spherical and all except the marine strain (D810), were phycocyanin-rich and formed blue-green coloured cultures and fluoresced red under epifluorescence microscopy. The strains' responses to cryopreservation, chemoheterotrophic growth, acetylene reduction assay, chromatic adaptation, motility and desiccation-tolerance were the same. No growth was detected in any strain following chemoheterotrophic incubation; no strain reduced acetylene to ethylene in aerobic, microaerobic or anoxic conditions; no strain was motile; no strain showed evidence of chromatic adaptation and no strain grew after rewetting desiccated cells. All strains grew successfully (though generally with long lag phases) when incubated in Chu 10 medium (+ NH_4-N 10 mg l^{-1}) following storage in liquid nitrogen. Strains showed greatest differences with respect to phosphatase activity.

Twelve out of 13 strains showed cell bound phosphomonestrase (PMEase) activity at pH 10.3 and all strains showed PMEase activity at pH 7.6. In all except 1 strain (D767) PMEase activity was greater at pH 10.3 than at pH 7.6. All 13 strains showed extracellular PMEase activity at pH 10.3 and all

Strain	Shape	Size (μm)	Synechococcus/Synechocystis	Fluorescence	Cryopreservation	Chemo-heterotrophic growth*	Acetylene reduction assay⊞	Chromatic adaptation	Motility	Desiccation tolerant⊞⊞	Cell bound PMEase pH 10.3 (μmol pNP mg d.wt^{-1} h^{-1})	Cell bound PMEase pH 7.6 (μmol pNP mg d.wt^{-1} h^{-1})	Cell-bound PDEase pH 10.3 (μmol pNP mg d.wt^{-1} h^{-1})	Cell bound PDEase pH 7.6 (μmol pNP mg d.wt^{-1} h^{-1})	Extracellular PMEase pH 10.3 (μmol pNP mg d.wt^{-1} h^{-1})	Extracellular PMEase pH 7.6 (μmol pNP mg d.wt^{-1} h^{-1})
D0033	elip	2	cocc	red	+	-	-	-	-	-	0.15	0.10	0.04	<0.04	0.64	0.06
D0562	rod	2	cocc	red	+	-	-	-	-	-	2.85	0.22	0.10	<0.04	9.45	0.61
D0767	elip	1.4	cocc	red	+	-	-	-	-	-	<0.04	0.10	0.06	<0.04	0.15	0.15
D0768	elip	1.2	cocc	red	+	-	-	-	-	-	0.42	0.14	0.08	<0.04	0.11	0.09
D0769	elip	1.8	cocc	red	+	-	-	-	-	-	0.42	0.26	0.12	<0.04	0.07	0.08
D0772	spher	1.6	cocc	red	+	-	-	-	-	-	0.55	0.13	0.06	<0.04	0.52	0.08
D0773	elip	2	cocc	red	+	-	-	-	-	-	0.22	0.07	<0.04	<0.04	0.10	0.10
D0774	spher	1.4	cocc	red	+	-	-	-	-	-	0.26	0.10	0.04	0.04	0.08	0.09
D0797	elip	1.2	cocc	red	+	-	-	-	-	-	0.22	0.10	0.10	0.04	0.31	0.08
D0798	elip	1.5	cocc	red	+	-	-	-	-	-	0.55	0.30	<0.04	<0.04	0.15	0.09
D0799	elip	1.5	cocc	red	+	-	-	-	-	-	0.25	0.10	0.08	<0.04	0.04	0.04
D0807	elip	1.7	cocc	red	+	-	-	-	-	-	1.45	0.20	<0.04	0.05	0.10	<0.04
D0810	elip	1.5	cocc	orange	+	-	-	-	-	-	ND	ND	ND	ND	ND	ND
D0838	rod	2	cocc	red	+	-	-	-	-	-	0.41	0.13	0.01	<0.04	<0.04	<0.04

* No detectable growth in glucose, sucrose, fructose, galactose, maltose or acetate.

⊞ No detectable acetylene reduction in oxic, micro-oxic or anoxic conditions.

⊞⊞ No growth following rewetting of cells grown on any substrate

ND No data

Table 7.3 Morphological and physiological differences between autotrophic picoplankton strains

but 1 strain (D807) showed extracellular PMEase activity at pH 7.6. 9 strains showed cell bound phosphodiesterase (PDEase) activity at pH 10.3, but activity was generally close to the limit of detection, and only 3 strains exhibited cell bound PDEase activity at pH 7.6. The highest activity of any strain was 9.45 μmol pNPP hydrolysed mg d.wt^{-1} h^{-1} (D562) in the extracellular PMEase at pH 10.3. D562 also had the highest cell bound PMEase at pH 10.3.

Table 7.4 Ratio of cellbound PMEase activity to extracellular PMEase activity at pH 10.3 and pH 7.6. Strains are ranked in increasing magnitude of the ratio (1 = highest)

Strain	Cell bound/extracellular PMEase activity			
	pH 10.3	Rank	pH 7.6	Rank
D033	0.2	12	1.6	6
D562	0.3	11	0.3	13
D767	0.1	13	0.6	12
D768	3.8	5	1.5	8
D769	6.0	4	3.2	3
D772	1.0	9	1.6	6
D773	2.2	8	0.7	11
D774	3.2	7	1.1	10
D797	0.7	10	1.2	9
D798	3.6	6	3.0	4
D799	6.2	3	2.5	5
D807	14.5	2	10.0	1
D838	20.5	1	6.5	2

There was over an order of magnitude difference between the lowest and the highest ratios in all strains at both pH 10.3 and pH 7.6. D838 had the highest ratio at pH 10.3 and the second highest at pH 7.6. D767 had the lowest ratio at pH 10.3 and the second lowest at pH 7.6. Four strains had higher ratios at pH 7.6 compared with pH 10.3.

7.44 Growth in organic substrates

As there were differences between strains in the amount of phosphatase activity, these studies were followed up by investigating the ability of the strains to utilize different organic phosphorus substrates. Strains were inoculated in Chu 10 medium (+ NH4-N 10 mg l^{-1}) from which inorganic phosphorus had been removed and organic substrates (pNPP, ATP, DNA, Bis pNPP and phytic acid) added to give a final concentration of 1 mg l^{-1} P. Strains were incubated at 32 °C in 100 μmol photon m^{-2} s^{-1} for 16 d after which cell density was enumerated. Controls were set up in Chu 10 medium with 1 mg l^{-1} inorganic phosphorus.

7.45 Results

The results are presented in Table 7.5.

Table 7.5 Yield of strains grown in five organic phosphorus sources (supplied as 1 mg l^{-1} phosphorus) for 16 days as % yield of strains grown in 1 mg l^{-1} inorganic phosphorus.

Strain	Organic phosphorus source				
	pNPP	ATP	DNA	Bis pNPP	Phytic acid
D033	78	120	106	0	0
D562	10	6	98	12	5
D767	72	89	15	0	15
D772	90	28	94	74	0
D773	77	41	92	34	0
D774	232	16	85	60	0
D797	151	0	46	76	0
D798	49	62	29	15	0
D799	80	133	84	45	0
D807	38	94	17	10	6
D838	63	20	74	26	5

Two strains (D774, D797) gave a higher final yield in pNPP than in

inorganic P and D033 and D799 gave a higher yield in ATP than in inorganic P. Phytic acid was generally the least utilizable phosphorus source. This might be simply a mechanical effect, resulting from the solubility of this phosphorus source. Experiments to improve growth conditions (shaking, stirring, aeration, prolonged incubation) gave no indication that strains could use phytic acid. There was no other substrate which had a similar broadscale response from the strains.

CHAPTER 8

DISCUSSION

8.1 DEVELOPING A CONSISTENT METHODOLOGY

The main practical difficulties in the study of picoplankton identified at the beginning of this study were i) how to define them, ii) how to separate them from other organisms in water samples and iii) how to count them. The definition in Sieburth et al. (1978) is very precise and clear cut, but in practice is not easy to adhere to. Defining a size cut-off helps researchers to gain a better understanding of the environment, but in nature size spectra are often continuous (see eg Murphy & Haugen, 1985). There is ambiguity, too, in the literature about the status of picoplankton-sized cells (sensu Sieburth et al., 1978) which are surrounded by mucilage and form colonies. Stockner and Antia (1986) include some of these organisms in their review of autotrophic picoplankton without critically justifying their inclusion. A recent paper by Stockner and Klut (1990) provides evidence for aggregations of viruses, autotrophic picoplankton, bacteria and larger phytoplankton in an oligotrophic lake and they suggest that "microaggregates" may lead to an underestimation of autotrophic picoplankton densities. The lack of precision in defining picoplankton is more than a point of academic debate because it impinges on the second problem in autotrophic picoplankton

studies: how to separate them from other organisms.

Most researchers use a filtration method to separate picoplankton cells from larger cells; some use 3-μm filters (eg Larsson & Hagstrom, 1982; Takahashi & Bienfang, 1983; Takahashi & Hori, 1984; Murphy & Haugen, 1985; Glover et al., 1985; Craig, 1984) and others 1-μm filters (eg Joint & Pomeroy, 1983; Li et al., 1983; Platt et al., 1983; Smith et al., 1985; Caron et al., 1985; Douglas, 1984). In all cases colonial forms and "microaggregates" would be retained by the filter and not form part of the counted picoplankton fraction. Depending on the filter used (3, 2 or 1 μm), different sized cells will be counted in the picoplankton fraction.

The third problem identified at the beginning of the study concerned counting the organisms using fluorescence. Most laboratories use an adaptation of the method devized by Hobbie and Daley (1977) in which cells are drawn onto membrane filters, mounted on glass slides and viewed under epifluorescence microscopy. The method is good for counting autotrophic cells, but there has been little effort to resolve the taxonomic status of the cells based on fluorescence characteristics. In particular few workers have attempted to distinguish prokaryotic from eukaryotic picoplankton in samples.

I used the definition of picoplankton as single, discrete cells in the size range 0.2 - 2.0 μm. This allowed the use of a prefiltration step to separate larger phytoplankton from the picoplankton fraction, which speeded up counting samples from mesotrophic and eutrophic waters where larger phytoplankton were often common. I selected a 3-μm prefilter which allowed cells larger than the upper limit to pass through rather than a 2-μm filter as Craig (1986) noted that some 2-μm cells are retained by 2-μm screens.

Measuring the cells with a graticule allowed me to exclude cells larger than 2 μm from autotrophic picoplankton counts. Although this method increased counting effort, it did at least ensure all cells were within the definitions of the size range. Cells in the 3-μm filtrate from the more oligotrophic waters were generally within the picoplankton size range and therefore the extra effort measuring cells was unnecessary. Cells in the 3-μm filtrate from the mesotrophic and eutrophic waters had a wider range of cell sizes and cells were frequently excluded from the autotrophic picoplankton count because they exceeded 2 μm.

An attempt was made to distinguish prokaryotric from eukaryotic autotrophic picoplankton cells (Chapter 3). A combination of methods were used: the fluorescence characteristics of cells excited by different filter sets (Section 3.32) and the fluorescence fading times of cells when viewed under epifluorescence microscopy (Section 3.4). After examining many samples it was clear that there were differences between cells. The most obvious difference was between red-fluorescing and orange-fluorescing cells. However, there were also differences between the red fluorescing cells. Some were uniformly red, others had a more intense fluorescence at the cell periphery. Also, some red cells fluoresced more intensely than others when viewed under green excitation light and others when excited by blue light. These observations were followed up with laboratory experiments to establish whether differences were between different taxonomic groups or just variations within a group of similar organisms. The results demonstrated that eukaryotic cells had very different fluorescence characteristics from those of autotrophic prokaryotic cells and that a distinction could be made between the two. However, the experiments were based on one culture of

Chlorella and three cultures of Synechococcus (Table 3.1) and further studies would be required before the findings could be extrapolated with confidence to field samples. Only a few authors have attempted a rudimentary and slightly subjective classification of autotrophic prokaryotes and eukaryotes using fluorescence characteristics (eg Craig, 1987; Weisse, 1988), but it seems that with a judicious choice of excitation filters, more information than simply numbers of autotrophic picoplankton can be obtained from epifluorescence microscopy. The colours of cells varies according to the filter sets and Glover (1985) has tabulated the optical filter combinations used by a number of workers and the corresponding colours of fluorescing cells. As Yentsch and Phinney (1985) suggest, the semi-taxonomic analysis of the size fractions via their pigment fluorescence adds a level of information to any study. I am unaware of any reported study relating fluorescence fading times to taxonomic group.

Another problem addressed in Chapter 3 was the preservation of cells (Section 3.2). This was an important study as several samples were collected from all over the world and counts were often made up to a month after sample collection. At the beginning of the study there was relatively little information on preserving autotrophic picoplankton. Several authors (eg Craig, 1986; Kuosa, 1988) preserved cells in glutaraldehyde or formalin for a day or two before counting, but most counted unpreserved material. Booth (1987) successfully stored marine Synechococcus in glutaraldehyde for two years and results in my study, although covering only one year, indicate that cells can be stored successfully in buffered glutaraldehyde or formalin (Fig. 3.1). Recent studies by Hall (1991) demonstrate that the best storage conditions for autotrophic picoplankton

cells are with 0.2% paraformaldehyde in the dark at -20 °C or -70 °C. She also reports that glutaraldehyde is not an effective preservative. An important difference between her studies and mine is that she stored cells on filters (ie slide preparations), whereas I preserved the water sample and prepared slides for counting at the time of counting. This may explain her lack of success with glutaraldehyde as there may have been an interaction between the preservative and the filter. Before Hall's (1991) recommendations are adopted widely, similar tests should be conducted on preparations with Nuclepore filters which are generally preferred to Millipore filters (used by Hall), for autotrophic picoplankton enumeration.

A major omission from my own preservation studies was the lack of an eukaryote. Bloem et al. (1986) reported a rapid loss of chlorophyll a fluorescence when glutaraldehyde was used as a fixative, and this could have led to a serious underestimation of autotrophic picoplankton from samples collected abroad, particularly as eukaryotic picoplankton are an important component of the autotrophic picoplankton community in some lakes (eg Malham Tarn in January and February, Fig. 5.3). Eukaryotic picoplankton were visible in my preserved samples collected from the lakes studied in Chapter 5, but enumeration was always within 24 h of collection. It is possible that cells preserved for much longer periods (like samples stored in the study in Chapter 4) suffered the loss of eukaryotes.

8.2 HOW WIDESPREAD ARE FRESHWATER AUTOTROPHIC PICOPLANKTON?

Establishing an effective preservation protocol enabled the temporal separation of sample collection and enumeration. This facility was used to

estimate autotrophic picoplankton populations from samples collected in the UK and from across the world.

Thirty samples were collected from waterbodies in the north of England and Scotland and twenty eight from twenty countries in five continents. The results suggest that autotrophic picoplankton may be ubiquitous: in every standing body of water sampled there were at least 100 autotrophic cells ml^{-1}. It is clear from the seasonal cycles of autotrophic picoplankton densities (Sections 1.6 and 5.2) that direct comparison of densities sampled at different times of year is impossible as densities may range within any lake by over two orders of magnitude. However, the range of autotrophic picoplankton from the samples collected from twenty countries (1.02×10^2 - 1.20×10^6 cells ml^{-1}, Table 4.1) is outside the range of the seasonal cycles in any of the UK lakes (Section 5.2).

None of the samples were from extreme environments and therefore claims of freshwater autotrophic picoplankton ubiquity in standing waters cannot be made confidently. Obvious omissions from the survey include samples from saline lakes and extremely acid environments. Reports of Aphanothece halophytica and Aphanocapsa salina in saline lakes are common (eg Dor & Hornoff, 1985; Ehrlich & Dor, 1985; Imhoff et al., 1979) though cells were not in the picoplankton size range (and they are colonial genera). Imhoff et al. (1979) record a Synechococcus from a saline lake in Egypt which they established in culture; unfortunately, the authors do not give the dimensions of the cells. There seems no reason per se why there should not be autotrophic picoplankton in saline lakes or acid waters although studies by Havens and Heath (1991) demonstrated that there was a shift from autotrophic picoplankton to net plankton in mesocosms which had been

acidified. Another omission from the samples gathered was temporary water bodies, such as puddles and some drainage ditches in which water may only be expected to remain for a period of days or even hours before draining away or evaporating. These environments are irrelevant when considering autotrophic picoplankton production in terms of global freshwater productivity, but should have been included in my survey to give a more accurate reflection of autotrophic picoplankton ubiquity. They are of interest, however, in establishing whether or not some picoplankton cyanobacteria can withstand desiccation, in view of the fact that none of the strains in culture exhibited this attribute (Table 7.2). Nevertheless, the study was valuable and provides the first published (Hawley & Whitton, 1990) survey of autotrophic picoplankton from a wide geographical area. Also, the survey goes a little way to gather autotrophic picoplankton data from countries highlighted by Stockner and Antia (1986) as being "virtually uninvestigated".

There are no published reports of autotrophic picoplankton in lotic waters so samples from the four rivers in northern England (R. Ehen, R. Liza, R. North Tyne, R. Tees) apparently form the first studies. Autotrophic picoplankton were only present in rivers in which a standing body of water was present in the catchment. In R. Ehen, R. North Tyne and R. Tees these are Ennerdale Water, Kielder Water and Cow Green reservoir respectively. The data set is too small to make general observations about autotrophic picoplankton in rivers, but in the four rivers studied it is likely that the standing waters provided the inoculum for cells in the river. Once the inoculum is supplied the flow and physical regime probably affect the ability of the autotrophic picoplankton population to increase. In the slow-flowing

reaches of R. North Tyne and R. Tees autotrophic picoplankton cell densities increased with increasing distance from the presumed source of inoculum. In the fast flowing and turbulent waters of R. Ehen, autotrophic picoplankton numbers decreased with increasing distance from Ennerdale Water. Cells in the R. Ehen were unusual in appearence compared with any other sample examined during the research project. Areas of cells were frequently devoid of fluorescence (Table 4.3). Aberrant cells may have been due to a number of factors: increased exposure to light in the shallow river may have caused photo-oxidation; cells may have been attacked by phage; or the loss of fluorescence may have been caused by mechanical damage from turbulence.

8.3 SEASONAL DENSITIES OF AUTOTROPHIC PICOPLANKTON

The aim of studies in Chapter 5 was to assess changes in autotrophic picoplankton populations in lakes with different trophic status. Based on the concentration of total chlorophyll, the ten lakes in this study may be considered to range from oligotrophic to slightly eutrophic (Table 5.3). They all resemble most previous studies of seasonal trends in autotrophic picoplankton (eg Krempin & Sullivan, 1981; Caron *et al.*, 1985; El Hag & Fogg, 1986; Joint *et al.*, 1986) in showing a maximum in mid- to late summer. The maximum value for autotrophic picoplankton density ranged from 2.7×10^3 (Esthwaite) to 1.06×10^6 cells ml^{-1} (Wastwater). All lakes showed temporal seasonal differences with respect to both autotrophic picoplankton density (Fig. 5.2) and relative abundance of cell type (Fig. 5.3). There was a range of total autotrophic picoplankton density in every lake of at least two orders of magnitude within an annual cycle. The maximum range was recorded in Ullswater with a difference between summer and winter densities of more

than three orders of magnitude. The minimum range was recorded in Wastwater with a difference of two orders of magnitude. Similar seasonal ranges in freshwaters are reported by Caron et al. (1985) (3 - 4 orders of magnitude in Lake Ontario), Nagata (1986) (ca 2 orders of magnitude in Lake Biwa) and Weisse (1988) (1 - 2 orders of magnitude in Lake Constance).

Two criteria are available for commenting on the trophic status of the lakes in my study: total filtrable phosphorus (measured in spring only, Table 5.1) and maximum biomass (chlorophyll a, Table 5.3) of the total phytoplankton. There were a negative relationship between values for the logarithm of maximum picoplankton density and both of the two measures of trophic status, the relationship being significant in the case of maximum chlorophyll a ($r = -0.6$; $p < 0.05$). Any interpretation of the data does however need to recognize that the two most eutrophic lakes also differed from the others in being much shallower. There was no indication of any relationship between the annual range in autotrophic picoplankton density and lake trophic status. Overall, autotrophic picoplankton density ranged during the year over about one order of magnitude more than total chlorophyll a, but individual lakes showed marked differences in this respect, ranging from Esthwaite (autotrophic picoplankton range : total chlorophyll range = 4) to Ennerdale (= 370). Other studies also show that autotrophic picoplankton biomass varies much more over the year than the total chlorophyll content of the water; in the north basin of Lake Biwa (Nagata, 1986) for example, the ratio between the two varies by at least a factor of ten.

Although direct measurements were not made of the contribution of the autotrophic picoplankton to total chlorophyll, approximate estimates can be made following Weisse (1988) and assuming cell density to be

1.07 g cm^{-3} and the dry weight content to be 30% of wet weight (Bakken & Olsen, 1983). If the assumption is made that chlorophyll is 1% dry weight, then the contribution of autotrophic picoplankton to the total chlorophyll at the time of maximum autotrophic picoplankton density is (μg ml^{-1}): Ennerdale, 3.6 x 10^{-3}; Wastwater, 2.1 x 10^{-3}; Thirlmere, 8.4 x 10^{-4}; Derwentwater, 1.0 x 10^{-3}; Malham Tarn, 1.8 x 10^{-4}; Bassenthwaite, 3.4 x 10^{-4}; Coniston, 2.5 x 10^{-3}; Cassop Pond, 1.0 x 10^{-3}; Ullswater, 2.0 x 10^{-3}, Esthwaite, 2.6 x 10^{-5}. Comparison with Table 5.3 shows that these values are in fact slightly higher than the observed values for chlorophyll in Ennerdale and Wastwater, suggesting either that the chlorophyll content of the autotrophic picoplankton was less than 1% or the direct measurements were slight underestimates; whichever is the explanation, it seems likely that the autotrophic picoplankton constitute most of the plankton in these two lakes in summer. The values for estimated autotrophic picoplankton chlorophyll as a percentage of total chlorophyll for the remaining lakes range from 46% in Thirlmere to 0.1% in Esthwaite. However, the work conducted in Wastwater by Vincent (1981) revealed populations of Cyclotella comensis, Peridinium pusillum, Kephyriopsis and a small Chlorella in the period mid-September to mid-November. Vincent's (1981) data and those in this study are not strictly comparable as the studies are separated by over a decade; nevertheless, the presence of phytoplankton larger than the picoplankton cannot be ignored and further investigations should focus on the contribution of different size fractions to total chlorophyll concentrations.

The percentage of phycoerythrin-rich autotrophic picoplankton out of the total autotrophic picoplankton showed several features: an increase in summer in all lakes, much higher percentage values in the deeper lakes and

the highest summer values in the most oligtrophic lakes. There was a sharp rise in this percentage value in all the lakes of the English Lake District in late winter or early spring, but as the time at which this occurred varied over several months, the process favouring this change is apparently not closely related to the development of a thermocline. In view of the fact that samples were taken at 0.5 m depth, it seems doubtful whether the light regime is the key factor leading to the success of phycoerythrin-rich, as opposed to phycocyanin-rich cyanobacteria. It seems possible that the phycoerythrin may have an important role as a nitrogen-store, as suggested for the marine environment by Carr and Wyman (1988). If this is true, then it is likely cells may be relatively phosphorus deficient, in which case various strategies may be adopted by cells to obtain the small amount of phosphorus in the environment. One possible strategy is the use of phosphatase enzyme to make the pool of organic phosphorus available to cells. Data from laboratory studies and comparison of values with other genera indicated that phosphatase activity is generally low in Synechococcus strains, though it is unfortuneate that no phosphatase data was obtained from waters in which phycoerythrin-rich cells were abundant.

As with most studies on lakes, those in the English Lake District have until recently largely overlooked the presence of autotrophic picoplankton. However, although they were not included in the account of "μ-algae" of Windermere, Blelham Tarn and Esthwaite (Lund, 1961), J.W.G. Lund (personal communication) using standard light microscopy, estimated the density of Synechococcus in 5-m tube samples from Buttermere, a mesotrophic lake, at various periods during 1960 - 1969. In 1960 and 1961, for which the most detailed counts were made, much higher values were found in spring than

summer. Studies using modern techniques are required to establish whether seasonal changes in the autotrophic picoplankton of this lake really do differ from those in other lakes in the region.

The ten lakes in the present study are similar to the majority of others previously reported in that the maximum autotrophic picoplankton density was in mid-to late summer. However it is unclear whether the response to temperature is direct, as suggested by Fogg (1986) for temperate oceans, or indirect, with grazing (Weisse, 1990), and nutrient concentrations and ratios also playing important roles. In situ experiments in Lake Constance combining a dilution technique and the use of a dialysis bag (Weisse, 1988) suggested that autotrophic picoplankton population dynamics were driven primarily by growth and grazing rates, with temperature less important. Synechococcus has been shown to predominate in oligotrophic lakes enriched with high N:P fertilizer (Stockner & Shortreed, 1988; Suttle & Harrison, 1988), but not in bioassays for the moderately eutrophic Calder Lake (Wehr, 1989).

Weisse (1988) concluded that the previous literature for lake and oceanic studies indicates that a positive relationship between the trophic state of waters and their autotrophic picoplankton abundance is likely, although his own data for Lake Constance gave values which were low in relationship to this scale; he suggested that the difference might be due to some colony-forming species with picoplankton-sized cells. My studies suggest the opposite to Weisse's conclusion: the highest autotrophic picoplankton densities apparently occur in large oligotrophic lakes (Table 5.4). However, there are several reports of dense Synechococcus populations in waters that are either known to be, or are probably highly eutrophic (eg

Drews et al., 1961; Bailey-Watts & Bindloss, 1968) and two examples of such populations in England during summer 1990 were brought to my attention. In at least some cases the cells of these single-celled populations from eutrophic waters were within the picoplankton size range.

Autotrophic picoplankton are likely to outcompete larger cells in nutrient poor waters, where their small size permits rapid uptake of dissolved solutes (Fogg, 1986). Vollenweider (1975) concluded from an examination of data from a number of European lakes that there was a systematic change in the ratio in the turnover velocities of nitrogen and phosphorus in passing from oligotrophic to eutrophic waters. Harris et al. (1983) and Harris (1984) have summarized the overall strategies of phytoplankton from oligotrophic and eutrophic waters and have shown that species from oligotrophic waters should be small, highly grazed and capable of rapid growth. In eutrophic waters, the cells should be larger, slower growing, relatively free from grazing and should be inefficient users of nutrients. Data from my studies fall in with these strategies: autotrophic picoplankton are more numerous and contribute a larger proportion of total phytoplankton biomass in oligotrophic waters compared with eutrophic waters (see page 127).

An aspect requiring more study before the relationship between autotrophic picoplankton density and lake type is clear is to quantify the occurrence of autotrophic picoplankton-sized cells loosely grouped together with mucilage. Examples of some of the more distinctive arrangements have been described by Cronberg and Weibull (1981) and Hickel (1981) from eutrophic lakes. Although such organisms have been regarded as picoplankton (Stockner & Antia, 1986; Stockner, 1988), they would be expected to be retained on a 3-μm filter.

A feature which differs markedly between the present studies and the studies in the meromictic lakes described by Craig (1987) is the size of the cells. Although Craig used a 3-μm filter, she reported the mean cell size to be 0.6 μm^3, a value also used by Weisse (1988) to estimate biomass. The data in Tables 5.4 and 5.5 indicate that most autotrophic picoplankton cells in this study are considerably larger than those found by Craig. Future studies should benefit from the more accurate data on size fractionation which can be provided by laser flow cytometry; it will also be important to study size changes and cell division over a daily cycle to establish how these influence estimates of cell volume. There will be a net increase in cell size following division and there may be significant cell size differences on a diel cycle: as carbohydrate is synthesized as a result of photosynthesis, cells may expand a little in the day time before cell division. Cells at the outer limit of the size range may expand sufficiently to be excluded by some filter fractioning protocols and therefore excluded from the picoplankton fraction in some studies.

8.4 VERTICAL DISTRIBUTION OF AUTOTROPHIC PICOPLANKTON

The vertical distribution of autotrophic picoplankton in the water column was studied at Kielder Water in July and August 1989 to determine whether or not densities were affected by a thermocline (Section 5.4). The results were not conclusive as thermoclines were present on both sampling dates but there was only a prominent difference between epilimnetic and hypolimnetic densities of autotrophic picoplankton in August (Fig. 5.4). Caron et al. (1985) found that densities of autotrophic picoplankton were three to four orders of magnitiude higher in the epilimnion compared with the hypolimnion

in Lake Ontario in August. Weisse's (1988) investigation of autotrophic picoplankton in Lake Constance revealed that during the period of lake stratification (mid-April to early October) autotrophic picoplankton densities were usually higher above the thermocline than below, although in late June and in early July highest abundances were measured at 12 - 16 m. In both these studies and others (eg Pick & Caron, 1987) autotrophic picoplankton densities were much higher in the epilimnion compared with densities in Kielder Water. Densities in Kielder Water were also lower at this time of year than in any of the ten lakes (Fig. 5.2) at similar times of the year. A reason for this may be the high humic content of the water and resultant shallow euphotic depth (Secchi depth was 2 m in August). Although no data are available, it is likely that autotrophic picoplankton were light limited. The chlorophyll a concentration in the epilimnion in Kielder Water in August were comparable to concentrations in Ennerdale Water and Wastwater, yet autotrophic picoplankton densities were about three orders of magnitiude lower in Kielder. Phosphorus concentrations are much higher in Kielder than in Ennerdale and Wastwater and this suggests the low phytoplankton biomass and low autotrophic picoplankton densities are light limited. At both sampling dates in Kielder Water, the mixing depth was lower than the euphotic depth so cells were probably below their compensation point for much of the time. Autotrophic picoplankton in the hypolimnion must have been in virtual darkness and may have been dead or dying. Conceivably it may have had a chemoheterotrophic mechanism, but this is unlikely as there is no evidence in from my own studies (Section 7.42) to suggest autotrophic picoplankton can grow in the darkness using an organic carbon source. White (1981) and Ellis and Stanford (1982) have investigated the occurrence and ecological

significance of algal heterotrophy in turbid, mesotrophic Lake Texoma, Oklahoma-Texas. Comparison of autotrophic, photoheterotrophic, and chemoheterotrophic activites of algal cells by microautoradiography revealed that algal heterotrophy occurred primarily in uncommon algae that were not particularly active autotrophs. White (1981) concluded that neither photoheterotrophy nor chemoheterotrophy were major sources of carbon for any phytoplankton in the Lake Texoma assemblage and heterotrophic carbon uptake by phytoplankton was negligible relative to photoautotrophy.

8.5 AUTOTROPHIC PICOPLANKTON DISTRIBUTION IN MICROHABITATS

Studies were made of autotrophic picoplankton densities in a variety of microhabitats in three waterbodies (Section 5.3). In general, greatest variation was found amongst replicates taken from marginal habitats. In Cassop Pond and Surha Tal, this was the weedy margins at the edges of the open water and in Ennerdale Water this was in the shallow areas. Marginal areas are more heterogeneous than open water sites due to the vegetation and influence of run off. Within a small area there may be gradients of light, temperature and nutrients, all of which could account for different growth rates of autotrophic picoplankton. Autotrophic picoplankton may be more susceptible to grazing in these areas as macrophytes can provide a refuge for zooplankton (Moss, 1987).

8.6 IN SITU GROWTH RATES

The aims of the studies in Chapter 6 were to assess the importance of grazers in controlling autotrophic picoplankton populations in nature and to measure autotrophic picoplankton growth rates in the absence of grazers. In

each of the experiments densities increased in the absence of > 3-μm grazers and growth rates were faster than in control incubations in which no grazers were removed (Fig. 6.1). The minimum doubling times in the 3-μm incubations ranged from 12.0 - 16.4 h (Table 6.1). These are similar to experiments reported by Bienfang and Takahashi (1983): 14.1 - 26.7 h, Bienfang et al. (1984): 9.6 - 34.2 h, Laws et al. (1984): 18.5 - 24 h using similar experimental methodologies.

A criticism of the method is that some grazers may not be removed by the filtration step. Protozooplankton are the main predators on bacteria (Sieburth, 1984) and they have been shown to consume and utilise autotrophic picoplankton for growth (Johnson et al., 1982). Many of these animals pass into 3- or 1-μm filtrates (Wright & Coffin, 1984) and there are even indications that 0.6- and 0.4-μm filters may not effectively retain them all (Fuhrman & McManus, 1984). Bienfang and Takahashi (1983) were concerned with this problem: they could not detect grazers in their samples but admitted that "difficulty in the detection/identification of small phagoflagellates means that their potential presence cannot be discounted altogether". A criticism of the method which seems to have been overlooked in other studies (and my own) is the possible exclusion of larger zooplankton (eg copepods) from the unfiltered control incubations. There is evidence that these zooplankton swim away from sampling vessels and therefore may not form part of the unfiltered control incubations but would be present in the ecosystem. Although there is evidence these larger zooplankton do not graze picoplankton (see Section 1.91), they may be responsible for preying on smaller zooplankton which do; therefore their absence from incubations could lead to an overestimation of grazing pressure on the

autotrophic picoplankton.

A possible alternative to removing the grazers is to inactivate them with eukaryotic inhibitors (eg Campbell, 1985; Sanders et al., 1985). The major difficulty with this approach appears to be that of inhibitor specificity ie the measured response may not necessarily correspond to that which is expected on the basis of the presumed primary effect of the inhibitor. Cycloheximide could be used to inhibit eukaryotic grazers although it would affect the eukaryotic component of the picoplankton too. The use of microautoradiographic methods (eg Itturiaga & Mitchell, 1986) may improve the identification and quantification of grazers. They found that a broad diversity of grazers ingest and effectively metabolize autotrophic picoplankton and most ranged in size from 10 - 50 μm.

Recently, Stockner and Klut (1990) provide evidence that phage may be an important factor in the control of autotrophic picoplankton. They found that the infection of Synechococcus by phage-like particles was most pronounced in late spring, when Synechococcus metabolic activity was high. However, Synechococcus abundance is low at this time of year, suggesting possible population control by phage. Suttle et al. (1990) concentrated viruses from sea water and added them to a growing culture of a marine autotrophic picoplankton and showed that within two days the population had crashed.

There was no evidence from my data to indicate phage played a major role controlling autotrophic picoplankton populations in the lakes. In the grazing experiments (Chapter 6), the 3-μm incubations grew at comparable doubling times to cultures in laboratory studies (Table 7.10) after accounting for temperature differences. However, the grazing incubations

were carried out at only one time of year and as Stockner (1990) suggests, phage may be important in the control of autotrophic picoplankton populations only at certain times of the year.

There was a possibility of phage attack in samples from R. Ehen (Table 4.3). Densities of autotrophic picoplankton decreased downstream from the presumed source of inoculum (Ennerdale Water) and there was also a decrease in the proportion of the cell which fluoresced. As far as I am aware there are no records in the literature describing aberrant autotrophic picoplankton cells and a possible explanation is phage attack. There are however other possible explanations for the unusual appearance of cells, including photoxidation of the pigment or physical damage from the turbulent water.

No attempt was made in my studies to check the presence of grazers in 3-μm incubations but identifying the major grazers is an area which requires further study, particularly in freshwater environments where less research has been conducted compared with the marine environment. One of the primary reasons for needing to identify autotrophic picoplankton grazers is to quantify "microbial looping" (Azam et al., 1985) in freshwater environments (Section 1.92). Identifying the organisms involved and quantifying their densities and activity will help to answer how much autotrophic picoplankton primary productivity is recycled within the microbial system and how much is transported to higher trophic levels.

8.7 COMPARISON OF STRAINS

The aims of the laboratory studies were to isolate as many strains as possible in clonal, axenic culture in order to test their growth in different conditions. 15 strains (Table 2.4) were used (though not all of them in all

studies) and of these, 9 were purified by myself.

A number of methods were used to purify strains (Section 2.8) All relied on plating on agar at some stage in the purification and this may have selected for a particular type of cell, particularly as strains did not grow vigorously on solid media. Waterbury et al. (1986) encountered similar problems when isolating marine Synechococcus strains. The easiest method of separating cells from bacterial contaminants was to use a flow cytometer and separate cells on the basis of fluorescence signals. Typically, cells needed to be sorted and re-sorted three times to produce an axenic culture (Section 2.824). As far as I am aware this is the first time flow cytometry has been used to clean laboratory cultures of algae although bacteria with genetic markers have been separated from natural populations using a similar method (R.W. Pickup, personal communication).

Several attempts were made to establish in culture cells from oligotrophic waters. These attempts failed and the collection of strains used in experiments was heavily biased towards phycocyanin-rich (red-fluorescing) cells isolated from mesotrophic or slightly eutrophic environments. It was unfortunate that the only culture of a phycoerythrin-rich (orange-fluorescing) organism was from the marine environment as these organisms were the most abundant in samples from all but the two shallowest lakes studied (Fig. 5.3). Failure to establish in culture phycoerythrin-rich cells from oligotrophic environments may have been due to several factors including contaminants in the medium and the build up of autoinhibitory substances. Brand (1986) reviews biases in culture collections and concludes (for marine collections) that they are heavily biased towards coastal organisms (roughly corresponding to enriched freshwaters) with very few oceanic representatives

(roughly corresponding to oligotrophic freshwaters); he gives the difficulties of culturing oceanic forms as the reason for this bias. The ability to store strains in liquid nitrogen (Table 7.2) reduces the chance of mutations from frequent subculturing, although the effects on the genetic material as a result of cryopreservation need to be investigated.

Waterbury and Rippka (1989) describe a new classification of Synechococcus, dividing <3-μm unicellular cyanobacterial cells into a variety of clusters (Section 1.5) on the basis of morphological, physiological and chemical properties and DNA base ratios. According to her classification there are two freshwater clusters, the Synechococcus-cluster and the Cyanobium-cluster; the former is represented by 10 strains and the latter by 8. Although the collection of strains used in my studies is relatively small compared with the 49 marine strains used by Waterbury et al. (1986), it is probably the largest number of freshwater strains isolated for a single series of studies. Also, it is a reasonable number compared with the number of strains used by Rippka (1986) to classify freshwater Synechococcus. Based on Rippka's (1986) classification, all the strains in my studies probably fit into the Cyanobium cluster: Cell size < 3 μm; phycocyanin-rich cells; no evidence of chemoheterotrophic growth, nitrogen-fixation or chromatic adaptation; cells non-motile. It is a pity my studies did not include measuring DNA base pair ratios, as this might have revealed a difference between strains.

Waterbury et al. (1985) showed that nitrogenase was inducible in a marine strain of picoplankton (Synechocystis); however, the strain was not axenic so nitrogen fixation has yet to be proven. Warterbury et al. (1986) tested 18 axenic marine autotrophic picoplankton strains for their ability to induce

nitrogenase and all were negative. In freshwaters, phosphorus is generally more limiting than nitrogen (Maitland, 1976) and therefore it is perhaps not surprising that freshwater strains are not nitrogen-fixers. Wyman et al. (1985) suggest that a proportion of the pycobiliproteins in picoplanktonic cyanobacteria are not used as light gathering pigments for photosynthesis but act as a nitrogen store; this further supports the argument that the need for nitrogen-fixing genes is redundant. However, the lack of nitrogenase activity using the acetylene reduction assay does not necessarily imply the absence of the nitrogenase gene and a molecular probe for the gene would be required to confirm this.

All the strains grew in ammonium, nitrate and urea as the nitrogen source in the medium (Fig. 7.1); in 6 of the 10 strains, growth rates were fastest in ammonium-nitrogen. It is widely accepted that all cyanobacteria can use nitrate- and ammonium-nitrogen for growth (Stanier & Cohen-Bazire, 1977; Carr & Wyman, 1986), though the possibility should not be ruled out that strains characteristic of particular environments may eventually be shown to lack the ability to use one of these substrates. A range of other inorganic and organic nitrogen sources has been shown to be utilized by various strains (see Carr & Wyman, 1986); All 49 strains of marine autotrophic picoplankton tested by Waterbury et al. (1986) utilized nitrate and ammonia as the sole nitrogen source for growth, but only about half used urea.

12 out of 14 strains tested exhibited cell-bound phosphomonoesterase (PMEase) activity at pH 10.3 and 9 out of 13 strains exhibited phosphodiesterase (PDEase) activity at pH 10.3 (Table 7.2). Most cyanobacteria can mobilize organic phosphates in their environment by means of cell-bound ("surface") phosphatase, which is usually an inducible activity

(Healey, 1982). However there are considerable differences between strains with respect to their abilites to hydrolyse organic phosphates in their environments. Whitton et al. (1991) tested 50 cyanobacterial strains (from 10 genera) for their ability to use organic phosphorus compounds. 7 Synechococcus strains from this study were included in the 50 (D033, D562, D767, D769, D797, D807, D838) and their phosphatase activity (both PMEase and PDEase) were amongst the lowest of all the strains studied. The ability of an organism to produce phosphatases allows it to utilize the pool of organic phosphorus; however the manafacture of any enzyme requires energy and picoplankton strains may rely on other strategies to sequester phosphorus eg high uptake rates on account of their small size (Fogg, 1986). Despite low phosphatase activities in relation to other genera (Whitton et al., 1991), the Synechococcus strains showed a range of values for phosphatase activity (Table 7.2). Highest phosphodiesterase activity was shown by D769, an isolate from a Bangladesh rice field. Four of the top five strains with detectable phosphodiesterase activity are from paddy or deepwater rice environments. Phosphodiesters, such as nucleotides, have been identified as major pools of dissolved organic phosphorus in various environments (Broberg & Persson, 1988), and these compounds may be a major organic phosphorus fraction available in deepwater rice and paddy habitats. There is a larger pool of total phosphorus in eutrophic compared with oligotrophic waters and the ratio of inorganic P to total P gets larger with increasing enrichment (Lean, 1973 a,b). Therefore it may be expected that phosphatase activity will be higher in oligotrophic waters compared with eutrophic waters. Generally, alkaline phosphatase activity (APA) increases with increasing trophic state (eg Jones, 1972) but APA normalised for chlorophyll declines

(Pick, 1987). The range in phosphatase activities in organisms in my studies may reflect the different environments with respect to phosphorus pools (both size and turnover times) from where they were isolated, but detailed water chemistry data would be required to comment critically on this. The fact that strains showed a wide range of responses to growth in various organic phosphorus sources (Table 7.3) indicates a possible difference in phosphorus metabolism strategies between strains and perhaps suggests that in enriched environments the ability to sequester phosphorus from a variety of different sources may give an organism an advantage over its competitors.

8.8 FUTURE DEVELOPMENTS

Much remains to be learned in the field of autotrophic picoplankton research. Knowledge of these plankters has progressed rapidly in the last decade, yet important questions remain unanswered: what is the mechanism of autotrophic picoplankton mobility? Are there picoplankton prochlorophytes in freshwaters? Do any autotrophic picoplankton possess nitrogen-fixing genes? Are grazers or phage the main control of population density or is nutrient limitation more important? Now that their widespread distribution has been established in most oceans and many freshwaters, the time is ripe to start answering more fundamental questions about the biology of the organisms. Studies in this thesis concentrate mainly on descriptive data concerning populations and has not addressed many aspects of autotrophic picoplankton biology. This can be justified in the light of the relative lack of knowledge concerning the distribution of freshwater autotrophic picoplankton at the outset of the project.

Despite the urge to leap ahead and unravel the intriquing mysteries of autotrophic picoplankton biology, workers in the field ought to pause to consolidate and resolve some of the problems in this area of research. In some respects autotrophic picoplankton research has progressed too quickly and not enough standardization of methodology and definition has been settled. An agreed working definition of autotrophic picoplankton should be established, particularly in the light of the discovery of "microaggregates". More work should be carried out to examine fluorescence characteristics of different cell types as taxonomic information gives a level of information which can augment simple estimates of densities. Much more effort needs to be directed towards culturing organisms representative of the natural phytoplankton assemblage, particularly from oligotrophic environments. Frequently, generalizations are made about organisms in the environment based on laboratory data from organisms which may be unrepresenative. By attempting to resolve some of these "basics" it is likely we will improve our understanding of this field of research, for example, we will have a better idea of the distribution of sizes of organisms in the <10-μm phytoplankton and we will have a better idea of the spectral signatures of cells under various conditions (an understanding of which is important when interpreting data from remote sensors).

Some of the properties of autotrophic picoplankton could perhaps be developed for use in the applied field of phycology. The widespread distribution, fast growth rates and relative ease of enumeration makes them good candidates for bioassays. Although Selenastrum capricornutum is widely used, autotrophic picoplankton could be used as complementary organisms. They may be particularly useful for assaying the influence of chemical or nutrient

additions to ultra-oligotrophic waters where the growth of a large species, such as a desmid, may be too slow for most purposes.

Summary

1. A methodology was devized to preserve autotrophic picoplankton for epifluorescence microscopy. Both 2% buffered (pH 7.0) glutaraldehyde or formalin were effective for storing cells in the dark at 4 °C for at least a year. Buffered formalin was used routinely throughout the research programme.

2. A method to distinguish cyanobacterial picoplankton from eukaryotic autotrophic picoplankton was developed. The method involved using different filter sets on a Nikon Fluophot microscope to distinguish chlorophyll a from accessory phycobiliprotein pigments. In addition a time course study of fluorescence intensities indicated that eukaryotic cells faded faster than cyanobacterial cells. A combination of these methods was used in a study of ten lakes to determine the relative abundances of eukaryotic and cyanobacterial cells in autotrophic picoplankton populations.

3. Studies were carried out to assess how widespread autotrophic piocplankton are in freshwaters. Samples were collected from standing freshwaters from 20 countries in five continents and all samples contained a minimum of 100 cells ml^{-1}. The highest density recorded was 1.20×10^6 cells ml^{-1} from Ennerdale Water in the UK. A more intensive study of UK standing waters was carried out in thirty lakes and ponds in Scotland and northern England. Densities ranged from 1.02×10^2 cells ml^{-1} in Esthwaite Water to 4.26×10^5 cells ml^{-1} in Ennerdale Water. Samples were collected from 4 rivers in northern England to determine whether or not

autotrophic picoplankton live in flowing water. In one river (R. Liza) no autotrophic picoplankton were found; in another (R.Ehen) atypical autotrophic picoplankton cells were found and decreased in number on passing downstream. The atypical cells were devoid of fluorescence in large parts of the cell. In R. North Tyne and R. Tees autotrophic picoplankton cells were not aberrant and increased in numbers on passing downstream.

4. A study of autotrophic picoplankton populations was carried out in ten lakes over a foutreen month period to examine seasonal cycles of densities. In every lake, densities reached a maximum in late summer and only in two lakes (Esthwaite Water and Coniston Water) were there spring peaks in autotrophic picoplankton. In every lake, the difference between minimum and maximum autotrophic picoplankton densities was at least an order of magnitude. Greatest densities of autotrophic picoplankton cells were found in oligotrophic waters and lowest densities in more eutrophic waters. The relative abundances of eukaryotic and cyanbacterial picoplankton cells varied from month to month in most lakes. The highest proportion of eukaryotic cells in the autotrophic picoplankton population was found in the two shallowest lakes (Cassop Pond and Malham Tarn) and in the deeper lakes phycoerythrin-rich cells were dominant in the summer but declined in relative abundance in the winter when phycocynain-rich cells became more abundant.

5. Densities of autotrophic picoplankton were studied in a variety of microhabitats in three standing waters and greatest coefficients of variation were found in the margins of each waterbody. The lowest coefficients of variation were generally in the open water. The vertical distribution of

autotrophic picoplankton was studied at a single site, Kielder Water. Samples were taken on two dates and on the latter there was a marked increase in autotrophic picoplankton density in the epilimnion compared with the hypolimnion.

6. Experiments were carried out to ascertain the effect of >3-μm grazers on autotrophic picoplankton populations. Experiments were carried out in three lakes: Bassenthwaite Lake, Surha Tal and Lake Gangebal. In each of the experiments, autotrophic picoplankton grew faster in the 3-μm filtered incubation compared with the unfiltered control. The results support the theory that autotrophic picoplankton populations are limited in nature by grazers.

7. Nine strains were isolated in axenic culture by the author and of these, eight were from water samples collected by himself or by colleagues. A number of different methods were used to clean the strains but the only successful ones were an aerosol method and the use of a cell sorter coupled to a laser flow cytometer. Autotrophic cells were separated from heterotrophic cells on the basis of red fluorescence and cell size. Only cells with a strong fluorescence signal were sorted. Generally 3 sorting operations were required to obtain uncontaminated autotrophic cells.

8. A comparison of the axenic strains was made with respect to morphological features, growth rates under different conditions (different nitrogen substrates and light conditions); enzyme assays (alkaline phosphatase assay and acetylene reduction assay); and other physiological

tests (desiccation tolerance, chemoheterotrophic growth, cryopreservation). The minimum doubling time of any strain under any condition ranged from 5.67 h to 24.00 h. Six of the strains had minimum doubling times when grown in standard conditions (ammonium nitrogen, 32 °C, 100 μmol m^{-2} s^{-1}) and the rest grew fastest when nitrate was the nitrogen source. Poorest growth was generally achieved with urea as the nitrogen source. Light and epifluorescence microscopy revealed some differences between strains, but all strains were similar in their response to cryopreservation (successful preservation for at least six months), chromatic adaptation (no evidence), motility (no evidence), acetylene reduction assay (no evidence) and desiccation (no evidence of tolerance).

9. Strains were different in their response to the alkaline phosphatase assay and growth in organic phosphorus substrates. The maximum activity of any strain was expressed by the extracellular phosphomonoesterase fraction of strain D562. Only three strains exhibited phosphodiesterase activity at pH 7.6 and in each case activity was very close to the detection limit. Of the organic phosphorus sources, phytic acid produced the lowest yield.

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